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*MUTAGENIC EFFECTS OF HIGH OXYGEN TENSIONS ON ESCHERICHIA COLI**

By W. O. Fenn, R. Gerschman, D. L. Gilbert, D. E. Terwilliger, and F. V. Cothran

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Communicated October 21, 1957

It has been postulated that there exists a common mechanism operating in the biological effects of oxygen and X-irradiation.[1] In further support of this theory it seemed worth while to determine whether high pressure of oxygen would cause mutations in *Escherichia coli* similar to those caused by radiation and demonstrated by Demerec.[2] Conger and Fairchild[3] demonstrated chromosome aberrations in *Tradescantia* caused by high oxygen pressures which were similar to those caused by X-rays. For this purpose Dr. Demerec kindly supplied us with a streptomycin-dependent strain (B/Sd-4) and furnished detailed descriptions of the method which he has employed.[4]

1. Heart infusion broth, containing 10 μg. streptomycin/ml, was inoculated with *E. coli* (B/Sd-4) and placed on a shaker for approximately 22 hours at 37° C.

2. After incubation, 100–150 ml. of the broth-bacterial culture were transferred to a centrifuge bottle. After centrifugation the supernatant was discarded, and the bacteria were washed in sterile saline to remove streptomycin and carefully resuspended in saline.

3. From this suspension, 0.15-ml. samples were spread on streptomycin-free agar plates for a count of the number of spontaneous mutants. From the same suspension a series of dilutions in sterile saline was made, each member of the series having one-fifth the concentration of the preceding one. Four 0.15-ml. aliquots of each of these dilutions (in the countable range) were spread on agar plates containing 10 μg. of streptomycin/ml. Colonies counted on these plates gave the number of viable bacteria of the streptomycin-dependent or "normal" strain prior to the experimental treatment.

4. Two 40-ml. samples of the suspension prepared under 2 were transferred to sterile centrifuge bottles or flasks which were placed in identical steel compression chambers at room temperature. One of these chambers was left at atmospheric pressure in air, while to the other was added pure oxygen to a total pressure of 4, 6, 8, or 10 atm. in addition to the oxygen present in the original air in the chamber. The latter chamber was left attached to the oxygen tank through a reducing gauge, so that the pressure was maintained at the desired level in spite

of small leaks during the night (about 16 hours). Generally, the temperature in the tanks was about 2° C. lower than the room temperature. After the exposure period the pressure was reduced slowly to avoid formation of bubbles.

5. Following the treatment, counts were made for both mutants and non-mutants in both samples according to the technique outlined in 3. Plates were incubated at 37° C. and counts were made after 24, 48, and 72 hours. All calculations were based on the final readings. Growth of the original B/Sd-4 strain was essentially complete after 24 hours, but only 20 per cent of the mutants had appeared in the same length of time. After 48 hours the growth of the mutants was about 90 per cent complete. Ryan[5] has described a similar delay in the appearance of mutants.

Results.—A preliminary series of experiments was carried out in the summer of 1955 (by D. T.). The first 3 experiments are omitted because of some differences in technique, and the remaining 19 experiments are summarized in Table 1. In

TABLE 1

COUNTS OF NORMAL (N) AND MUTANT (M) FORMS OF *E. coli* AFTER TREATMENT WITH 6–10 ATM. O_2 FOR 16 HOURS AS COMPARED TO TREATMENT WITH 1 ATM. OF AIR FOR SAME PERIOD

(Averages of 19 Experiments, with Standard Errors)

	$N \times 10^8$	M	$M/N \times 10^{-8}$	$\frac{M/N \text{ in } O_2}{M/N \text{ in Air}}$
Air	16.6	43.4	3.4 ± 0.055	1.55 ± 0.16
O_2	14.8	47.1	4.7 ± 0.86	

this series no counts were made on the suspension prior to treatment, so the table includes only the counts made on the suspensions after exposure to air or 6–10 atm. of O_2 for about 16 hours. Actual counts are given for the number of normal streptomycin dependent (N) and for the mutant or streptomycin-independent (M) strains, as well as the number of mutants per 10^8 normal bacteria (M/N). It is seen that the number of mutants per 10^8 of viable bacteria increased from 3.4 to 4.7 as a result of the oxygen treatment. While the difference between these two average figures is not significant, on account of the wide variations between the densities of the suspensions in the different experiments, a significant result is obtained by calculating for each experiment the ratio of the M/N value for O_2 to the M/N value for air. The average result in the last column shows that the treatment with oxygen increased the number of mutants per 10^8 viable bacteria 1.55 ± 0.16 times. This value differs from 1 with a $P = 0.3$ per cent.

Treatment with oxygen tended to kill or prevent the growth of the normal strain by 10 per cent ($N_{O_2}/N_{air} = 0.90$), while it increased the growth of the mutant strain by 25 per cent ($M_{O_2}/M_{air} = 1.25$). These differences are significant at the 5 per cent and 2.2 per cent levels, respectively. Decreased growth of the normal strain is only partly responsible for the increased number of mutants in O_2 per 10^8 of viable bacteria. The data suggest also an absolute increase in the number of mutants as a result of the oxygen treatment.

A second series of experiments was carried out in the spring of 1956 by B. Ruhm and D. Caccamise. The purpose of these experiments was to demonstrate more clearly that the oxygen produced an absolute increase in the number of mutants. To this end, counts were made in the suspension *before* it was exposed to oxygen

or air in addition to the counts made afterward. The results in Table 2 show that the number of normal streptomycin-dependent bacteria (N) was little affected by treatment with either air or oxygen but the number of mutants (M) was increased from 66.7 to 129.5 per ml. by treatment with oxygen, while similar treatment with air had no appreciable effect. This is the essential finding, but the results can be analyzed in other ways to the same end. Thus the figures show that the number of mutants per 10^8 of normal bacteria (M/N) was increased on the average 2.44 ± 0.47 times ($P = 0.016$) by O_2 treatment as compared to air. This figure compares with the value of 1.55 ± 0.16 obtained from Table 1. The count of the normal bacteria was increased 16 per cent after treatment in air (perhaps due to additional growth) and was decreased 7 per cent by treatment with 10 atm. of oxygen. The number of mutants was decreased 5 per cent by air treatment (relative to the pretreatment control) but was increased 93 per cent by treatment with oxygen.

TABLE 2

COUNTS OF MUTANTS (M) AND NONMUTANT (N) FORMS OF *E. coli* BEFORE TREATMENT AND AFTER EXPOSURE TO AIR OR 10 ATM. OF O_2 FOR 16 HOURS

	Before			After Air			After O_2			
Exp. No.	N ($\times 10^8$ per Ml.)	M (Per Ml.)	M/N ($\times 10^{-8}$)	N ($\times 10^8$ per Ml.)	M (Per Ml.)	M/N ($\times 10^{-8}$)	N ($\times 10^8$ per Ml.)	M (Per Ml.)	M/N ($\times 10^{-8}$)	$\frac{(M/N)\ O_2}{(M/N)\ \text{Air}}$
1	34.0	41.7	1.23	24.1	36.7	1.52	23.5	81.7	3.48	2.29
2	24.1	78.3	3.24	29.6	78.3	2.64	16.6	162.2	9.77	3.70
3	32.8	75.3	2.30	45.8	96.7	2.11	37.6	431.3	11.50	5.45
4	37.6	61.7	1.64	51.9	40.0	0.77	42.8	105.0	2.45	3.18
5	23.7	58.3	2.46	30.6	78.0	2.55	24.7	118.7	4.80	1.88
6	14.7	53.3	3.63	14.8	53.2	3.80	9.5	65.0	6.84	1.80
7	26.7	56.7	2.12	9.0	46.7	5.19	20.8	61.3	2.95	0.57
8	13.1	73.7	5.60	20.7	75.3	3.63	16.0	53.3	3.33	0.92
9	15.4	101.3	6.58	22.6	56.7	2.51	15.7	86.7	5.52	2.20
Av.	24.7	66.7	3.20	27.7	62.4	2.75	23.0	129.5	5.63	2.44
St. error	±2.8	±5.5	±1.25	±4.2	±6.6	±0.4	±3.4	±0.37	±1.0	±0.47

The arbitrary selection of 6–10 atm. for the experimental O_2 pressure proved fortunate, since higher pressures are progressively more toxic. In 4 additional experiments by J. V. C., counts were made before and after treatment for 16 hours. The normal count was reduced to 54 per cent after exposure to oxygen, while the mutant count rose to 197 per cent. The M/N ratios were, of course, all increased after the treatment with oxygen, but this was partly due to some killing of the normals by oxygen. Nevertheless, the absolute number of mutants was increased by the O_2 treatment.

It must be admitted that there are considerable variations among the different experiments so far presented. Oxygen appeared to increase the number of mutants in 14 out of 19 experiments in Table 1 and in 7 of the 9 experiments in Table 2. Considerable errors are to be expected in a technique of this sort, however, and statistically the data show clearly that high pressure of oxygen does have a mutagenic effect.

The possibility still remains that the data presented may depend upon pressure per se rather than upon the partial pressure of the oxygen itself. To answer this question, we have tried a number of experiments with nitrogen in place of oxygen, and these results are presented in Table 3. The first 3 experiments were done

TABLE 3

NITROGEN CONTROLS: COUNTS OF NORMAL (N) AND MUTANT (M) FORMS OF *E. coli* BEFORE AND AFTER TREATMENT WITH AIR OR AIR PLUS 10 ATM. N_2
(Average Values and Standard Errors)

	N ($\times 10^8$)	M	M/N ($\times 10^{-8}$)
3 experiments:			
After air	14.6 ± 2.0	33.4 ± 6.2	2.25 ± 0.14
After N_2 + air	16.4 ± 2.5	22.7 ± 2.2	1.48 ± 0.13
6 experiments:			
Before treatment	29.1 ± 2.0	53.6 ± 5.0	1.89 ± 0.24
After air	28.0 ± 3.0	59.1 ± 7.8	2.15 ± 0.25
After N_2 + air	25.4 ± 2.8	62.0 ± 7.5	2.54 ± 0.31

along with the experiments of Table 1 and lacked a count prior to the overnight treatment with air or nitrogen (10 atm.) plus air. In this case the nitrogen caused, if anything, a decrease in the number of mutants. This may, however, have been due to anoxia, since the chambers leaked to some extent and allowed the nitrogen to flush out some of the oxygen originally present in the chamber. Another 6 experiments were carried out later in which 2–3 per cent of oxygen was added to the nitrogen to avoid this complication. The averages of these experiments show no significant difference between the bacteria exposed to air alone and those exposed to air plus 10 atm. of nitrogen. Likewise, the counts are the same before and after treatment within the limits of the experimental error. It is concluded that pressure per se does not influence mutagenesis.

It has been shown by Jordan, Mefferd, and Wyss[6] that *E. coli* can be protected against the toxic effects of radiation by treatment with pyruvate. Gerschman, Gilbert, *et al.*[1] have shown that certain substances which protect against radiation are also protective against oxygen poisoning. For this reason we tried adding pyruvate to our cultures of *E. coli* to find out whether this prevented the mutagenic effect of high oxygen pressures. No counts prior to treatment were made, but 3 suspensions were set up: (1) without pyruvate but exposed to oxygen, (2) with pyruvate and exposed to oxygen, and (3) with pyruvate but exposed only to air. The average results of 6 experiments (by F. V. C) are shown in Table 4. Com-

TABLE 4

ELIMINATION OF THE MUTAGENIC EFFECT OF OXYGEN BY PYRUVATE*

	N (Per Ml. $\times 10^8$)	M (Per Ml.)	M/N (Per Ml. $\times 10^{-8}$)
Oxygen	26.3 ± 4.3	156 ± 37	6.1 ± 1.5
Oxygen + pyruvate	16.4 ± 3.7	38.3 ± 5.6	2.9 ± 0.7
Air + pyruvate	28.0 ± 3.2	55.6 ± 8.7	2.2 ± 0.4

* Counts of normal (N) and mutant (M) forms of *E. coli* after treatment with O_2, with and without pyruvate and after air + pyruvate. Average of 6 experiments with standard errors.

paring suspensions 1 and 2, it is evident that the addition of pyruvate to cultures exposed to oxygen diminished the M/N ratio from 6.1 to 2.9. Comparing suspensions 2 and 3, it appears that the exposure to oxygen instead of air in cultures containing pyruvate caused no significant increase in the M/N ratio. It is concluded from these figures that pyruvate prevents the mutagenic effect of oxygen, just as it decreases the mutagenic effect of radiation in the same organism. Many more experiments of this type are required, but these preliminary results encourage us to believe that further experimentation will support the thesis that there is a certain similarity of fundamental mechanism between radiation and oxygen toxicity.

Discussion.—The experiments reported appear to show that high pressures of oxygen are capable of exerting a mutagenic effect in *E. coli* which is comparable to the mutagenic effect of radiation or of chemical mutagenic agents such as those described by Demerec[2] and Demerec, Bertani, and Flint.[4] Conversely, these same mutations are inhibited by anoxia.[7, 8] As a possible explanation for this similarity between oxygen and radiation, one thinks of chromosome damage by free radicals formed from the effects of radiation on water or formed by high pressures of oxygen in a biological medium where univalent oxidations are taking place.[9] According to Giles,[12] oxygen increases the mutagenic effect of radiation only when it is present during the time of the radiation and because it intensifies the amount of chromosome breakage. Baker and Von Halle,[11] however, believe that oxygen affects the result because it interferes with the subsequent union of the broken ends of the chromosomes. In neither of these papers is there any discussion of the effects of oxygen by itself without any simultaneous radiation. According to Plaine,[10] exposure to oxygen alone produces a condition known as "erupt eyes" and increases the incidence of tumors in *Drosophila melanogaster*. The effect of radiation in producing these same abnormalities is then regarded as "an enhancement of the effect of oxygen tension alone" and is ascribed to the formation of hydrogen peroxide. Formation of free radicals might have a similar effect. On the other hand, Lück[13] has found that treatment of bacteria with compressed oxygen causes increased catalase activity and inhibition of alkaline phosphatase, dehydrogenase, and rate of formation of lactic acid and CO_2. All these enzymic changes might, however, be regarded as secondary to some primary effect such as the formation of free radicals. Holman[14] suggests that the formation of tumors is analogous to the formation of abnormal bacteria and attributes the result to an increase in the concentration of hydrogen peroxide.

In reviewing this work Dr. E. A. Adelburg has pointed out to us the possibility of selective survival in oxygen as another reasonable explanation of our observations. As a result of this suggestion, we tried further experiments with additional counts made prior to the exposure to oxygen. The results show that selective survival is not the only explanation of the figures because we have now demonstrated an *absolute increase in the number of mutants* after exposure to the compressed oxygen as compared to the same culture before treatment. Nevertheless, there is perhaps still room for some reservation. It is conceivable, for example, that the cells might come in clumps and that each clump might act either as a normal or as a mutant, but not both. Then if oxygen tended to kill off the normal cells, more of the clumps might function as mutants. Similarly, each individual cell might have the equivalent of more than one nucleus, some of them normal and some mutant. Oxygen might then promote the selective survival of the mutant "nuclei" and so result in more cells under mutant than under normal control. It might also be supposed that oxygen would tend to unclump more mutants than normal cells, but such highly selective action seems highly improbable. Except for such rather remote possibilities, our experiments appear to demonstrate a true mutagenic effect of oxygen, due presumably to chromosome damage resulting from an increased concentration of free radicals.

Summary.—Using a streptomycin-dependent strain of *E. coli*, it is shown that the number of spontaneous mutants to the streptomycin-independent variety is

increased 1.5–2.4 times by treatment for 16 hours with 6–10 atm. of oxygen. This demonstrates a mutagenic effect of high pressures of oxygen which is comparable to that caused by radiation.

We are indebted to Dr. E. A. Adelberg, Dr. A. H. Doermann, Dr. M. Demerec, Dr. A. W. Ravin, and the senior author's daughter, Dr. Priscilla F. Roslansky, for critical reviews of this report. We acknowledge also the excellent technical assistance in some of the work of Mr. Barclay Ruhm and Mr. Daniel Caccamise.

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*MOLECULAR STRIAE FROM AN INDANTHRENE DYE**

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It has recently been demonstrated by Menter[1] that electron micrographs of suitably oriented crystals of copper and platinum phthalocyanins may appear covered by striations separated by the known distance between molecular centers. These observations have also provided a striking demonstration of dislocations and other crystalline faults at the molecular level and are beginning to yield valuable information concerning the interaction between a coherent beam of electrons and a crystalline sample under the conditions requisite to high-resolution electron microscopy. In view of the wide potential usefulness of so direct a method of visualizing the separations of molecules having weights of a few hundreds, we have been examining a number of crystalline organic substances of suitable molecular size, to define more clearly the conditions under which these phenomena appear and to see how they differ from compound to compound. Such observations are needed to

provide the data for an adequate detailed interpretation of what is seen and to indicate the types of compound that can be studied most profitably.

At the outset of this investigation[2] a comparison was made between phthalocyanin itself and its copper compound; this demonstrated that, given crystals of suitable dimensions, the presence of heavy atoms in the molecule is not necessary for the production of good striations. The wider range of compounds that thus become worth examining probably have two properties evident in the phthalocyanins. One is a high sublimation temperature, indicative of a thermal stability sufficient to withstand the intense electron bombardment involved in the photography of their crystals. The other is a platy character to their molecules. Assuming that the observed striae do indeed represent molecular separations, they should best be developed from crystals with molecules that stack with a minimum of confused overlay; crystal-structure data show that large platy molecules often yield such crystals.

Because many of the commercially available indanthrene dyes have such large flat molecules and high thermal stabilities, they were chosen as a promising group of substances for the exploratory work of this laboratory. The first to be studied in detail—indanthrene scarlet R ($C_{38}H_{22}N_2O_6$)—has given the results to be described here.

The crystalline preparations for electron microscopy were made by vacuum sublimation of the dye directly onto conventional copper grids. Under suitable conditions, single crystals in the form of minute needles no more than 200–300 A thick will grow out into the meshes of these grids. They can be observed there without the substrate that would be required if preformed crystals were used. Most of the crystals growing on such a grid are, of course, not oriented to exhibit striations, but suitable individuals are found if enough preparations are examined. Approximately two hundred crystals showing striae have been photographed in the study of this dye.

The electron microscope used has been an early RCA EMU-2 instrument modified in this laboratory to give improved resolution and equipped with an auxiliary power supply to permit photography at an initial magnification of 50,000×. A magnification at least as high as this is essential if phenomena of the order of 20 A or less are to be seen with certainty against the confusion produced by photographic grain. The successful recording of crystalline striations with this instrument has been rendered difficult by the rapidity with which an obscuring layer of contamination builds up on specimens exposed to the electron beam. Since this accumulates at a rate of about 200 A per minute in a beam of the necessary intensity, all photographs must be made within less than 1 minute's total exposure of a crystal to the beam.

The series of striations photographed with crystals of indanthrene scarlet R are more complex than those seen with phthalocyanin. Three characteristic separations, with the spacings 15.4, 19.3, and 28.1 A, have been found. Crystals showing the 19.3 A spacing (Fig. 1) have closely resembled the phthalocyanin crystals in being covered by a pattern of black and white stripes of uniform width. Many of the 15.4 A patterns are equally uniform (Fig. 2, *a*), but, as the other two photographs of this figure show, this is not always the case. In Figure 2, *b*, alternate dark stripes are especially black, while in Figure 2, *c*, alternate light bands represent

FIG. 1.—An electron micrograph of a crystal of indanthrene scarlet R covered with parallel striae separated from one another by 19.3 A. The light and dark bands are of approximately equal width. The unstriated region around the periphery is contamination which has been deposited during the short period of examination. Many details in this and the succeeding photographs are best seen by looking at them along the direction of the striations at an almost glancing angle. Magnification 1,060,000×.

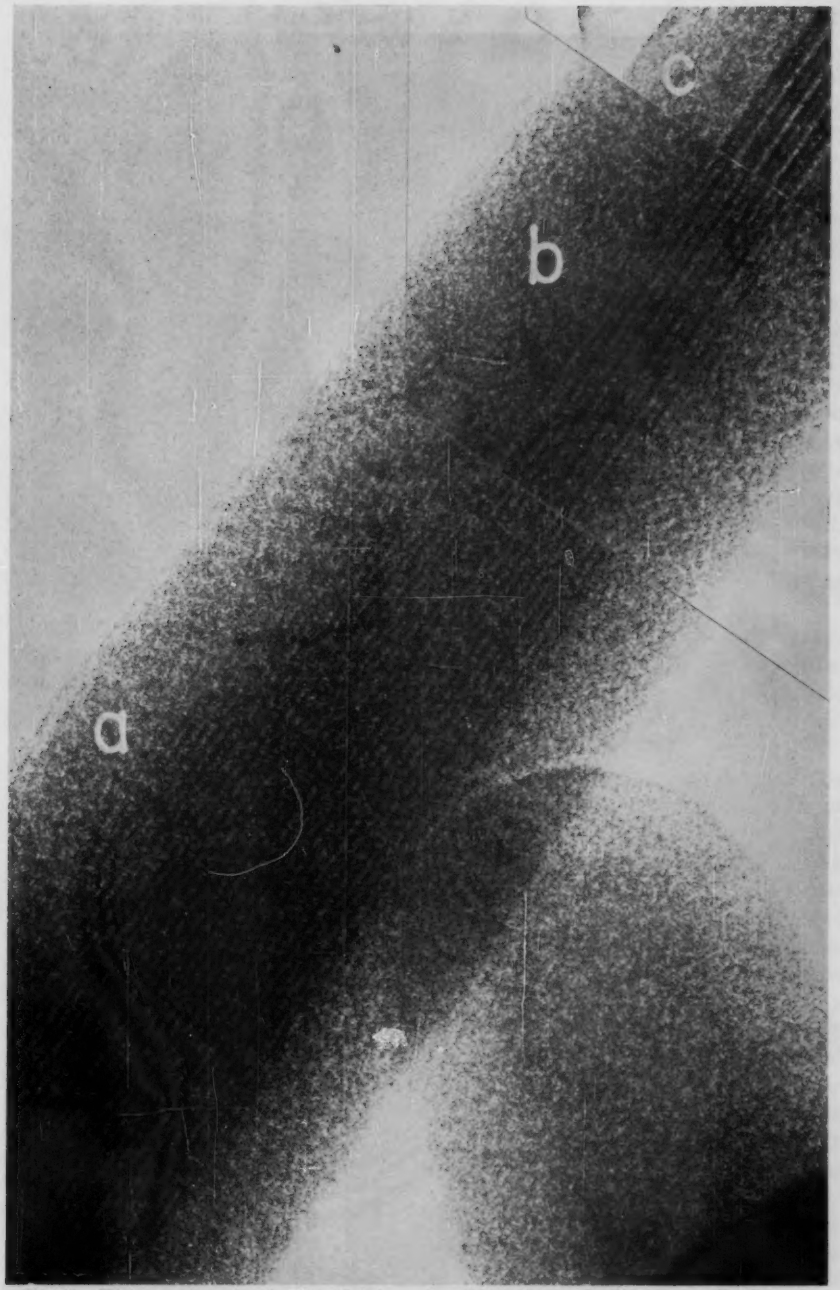


FIG. 2.—Three crystals showing the 15.4 A system of striations. In the crystal of *a* the striae, like those of Figure 1, consist of uniform light and dark bands of equal width. In crystal *b* alternate dark bands are blacker than in others. In *c* alternate light bands are regions of especial transparency. 1,060,000×.

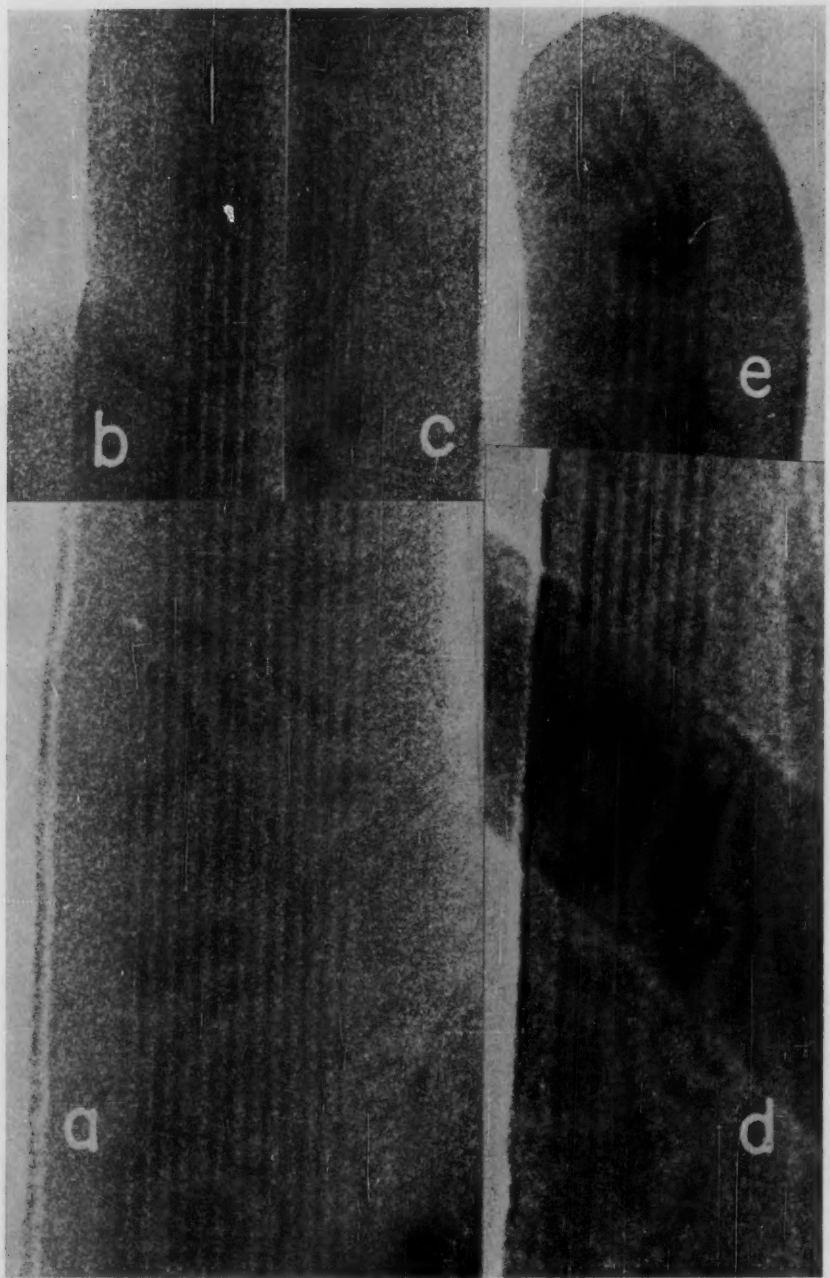


FIG. 3.—Crystals showing various aspects of the 28.0 A system of striae. In *a*, *d*, and *e* the dark bands are broad and unresolved. In *c* the splitting into a succession of 14.0 A striae is clear; in *b* the splitting, just apparent, is best seen by looking along the striae. 1,060,000×.

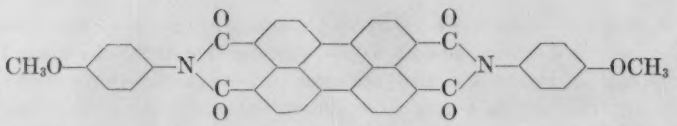
regions of especial transparency. The 28.1 spacings are no less complicated. As seen in Figure 3, *a*, *d*, and *e*, the dark bands are wider than those of Figures 1 and 2. Occasionally, as in Figure 3, *c*, they are more or less completely resolved to yield separations of 14.0 A; in Figure 3, *b*, this splitting, though perceptible, is less developed. Figure 3, *e*, is also interesting, in showing near the top of its crystal a twinning that leaves the striae uninterrupted but sharply changed in direction. The 14.0 and 15.4 A spacings differ by so little that a careful check was made to assure their distinctness; they can be distinguished with certainty. The intermediate 19.3 A pattern of striae has shown none of the complexity of the other two; it has always shown light and dark bands of approximately equal breadth (Fig. 1).

The photographs made during the study of this dye have been sufficiently numerous to permit measurements of the constancy of the separations as observed from a given substance. Thus, in one set of data on 18 plates showing the 15.4 A spacing, the average variation is 1.4 per cent, and the maximum departure from the mean 3 per cent; in a similar determination of the intermediate spacing, the average value is 19.3 A $\pm$ 1.3 per cent, and the maximum deviation from this is 2.5 per cent. The largest spacing is 28.0 A $\pm$ 3.1 per cent. These separations thus are strictly constant within the limits of experimental error and give no indication of varying with small changes in crystal angle about an optimal position.

Three other indanthrene dyes have been examined. Two have yielded crystals that show striae; crystals of the third have always been too thick. Their separations, which reach down to 9 A (the lower limit that can be resolved by the available experimental arrangement), will be reported in detail when the examination has been completed.

It is obviously of the greatest importance to be sure of the interpretation to be given these striae. In the case of the phthalocyanins, the measured separations (of which there are two) agree so well with those to be predicted from X-ray diffraction studies that it is attractive to consider them qualitatively as views down crystals so oriented as to reveal molecular separations. It is natural to assume, as Menter has done and as is consistent with the theoretical treatment of Niehrs[3] of phenomena to be expected from crystals of MgO, that one sees a composite of the zero-order image and the few crystalline diffractions formed and passed by the severely apertured electron optical system of the microscope. In this sense, it may be convenient to think of the electron image as a very incomplete Fourier projection of the molecular structure of the crystal produced by the automatic summation of the few crystalline reflections that can contribute to it. Very evidently, the striae are phenomena of interference. They critically depend for their appearance on both the orientation and the thickness of the crystal, and the photographs shown here demonstrate that they are as clear in micrographs taken on either side of focus as in pictures made at exact focus.

The X-ray diffraction data needed for a more detailed comparison between striae and structure are not yet available for indanthrene scarlet R. Measurements leading to the determination of the unit cell have, however, been undertaken, and these will permit such comparisons. Nevertheless, it is interesting to see how the observed spacings compare with the probable length of the molecule. This dye has the following formula:



Crystal-structure studies of complex organic compounds indicate that the central part at least of such a molecule is planar; hence it is simple to calculate from these data the probable distance between molecular centers in the direction of the long axis. It proves to be 31.5 A.

It is important to distinguish between these striations and superficially analogous diffraction phenomena that have hitherto been observed in the electron microscope. These have been of two sorts. One is the system of equi-spaced bands to be seen covering minute MgO crystals viewed in an unapertured microscope.[4] The separations of these bands may be uniform for one preparation, but, unlike the phenomena considered here, they will vary by as much as 100 per cent from preparation to preparation. Their separations are of a higher order of magnitude (200–300 A), and they shift position over the image of the crystal with change in objective lens current; undoubtedly they, but not the present phenomena, are diffraction effects produced by a crystal that is wedge-shaped. Crystals of MoO_3 as studied by many microscopists give the best example of the other type of banding. These are usually much broader and less sharp and are produced by overlying crystals and not by single individuals. Under electron bombardment, crystals of MoO_3 visibly alter and warp; the bands develop as this change is going on and form a moiré pattern produced by diffraction taking place in superimposed crystals slightly displaced by this warping. As such, they are unrelated to the striae that are the object of the present study.

Summary.—High-resolution electron micrographs show suitably oriented, very thin crystals of the dye indanthrene scarlet R, $C_{38}H_{22}N_2O_6$, to be covered with patterns of striae analogous to those recently described by Menter from the phthalocyanins. Measurements of these striae are recorded, and their nature is briefly discussed.

* A paper read April 23, 1957, before the National Academy of Sciences.

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THE INCORPORATION OF C^{14}-ADENINE AND C^{14}-PHENYLALANINE BY DEVELOPING ROOT-TIP CELLS

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The combination of morphological and biochemical analyses of sections of the root tip has resulted in the general characterization of the early stages of cell devel-

opment in the root. The techniques used have limited the data obtained to average per cell figures and indirect correlations. The use of labeled precursors and autoradiographic techniques permits a more direct study of cell development. This approach has demonstrated the direct relation of ribonucleic acid (RNA) to amino acid incorporation[1] and has also shown the existence of a group of cells in the root tip which have a low rate of deoxyribonucleic acid (DNA) synthesis.[2] The present work employs the first 2 mm. of the onion root tip, which has been analyzed morphologically[3] and to a limited extent biochemically,[4] and is concerned with the relation of the incorporation of C^{14}-adenine and C^{14}-phenylalanine to cell division and development. The effect of ribonuclease (RNA-ase) treatment of the roots on subsequent incorporation of these compounds has also been investigated.

Materials and Methods.—Sets of *Allium cepa* var. White Globe were used as the source of all roots. The sets were placed, base down, on vials of tap water, and roots appeared in 2 or 3 days. The roots were kept relatively dark, while the bulbs were allowed the normal day-night variations of light and temperature. Under these conditions there is a marked periodicity of cell division, with maxima occurring at 11:00–1:00 over both noon and midnight. The C^{14} compounds were presented to the roots at either 11:30–2:30 or 2:30–5:30. These intervals represent the approximate maximum and minimum periods of cell division; the 11:30–2:30 period, however, is representative of the later stages of division and the reconstituting nucleus.

The roots used were 10–15 mm. long and were carefully selected for uniformity. The roots were excised and immediately placed for $^1/_2$, 1, or 3 hours in C^{14}-phenylalanine (0.1 μC/ml) or C^{14}-adenine (0.5 μC/ml). After preliminary experiments, only the 3-hour period was used, since this time period at this concentration of radioisotope gave the best counting conditions. The roots were then washed and frozen in isopentane cooled with liquid nitrogen. The freeze-substitution method of dehydration[5] was used, and the roots were paraffin-infiltrated and imbedded. The first 2 mm. of the root were sectioned transversely at 10 μ and mounted on slides. The paraffin was removed with toluene, the sections passed through absolute and 95 per cent alcohol and air-dried. They were then placed in inactive adenine or phenylalanine for 1 hour, washed briefly in distilled water, passed through 95 per cent alcohol, and air-dried. Autoradiographs were made by the liquid-emulsion procedure of Ficq.[6] Three-day exposures were used, and after development the sections were stained with Unna's pyronin-methyl green combination. Some adenine-treated roots were always treated with ribonuclease (1 mg/ml) before the autoradiographs were made, to distinguish between incorporation of adenine into RNA and DNA. In all cases, virtually all activity was removed by the RNA-ase treatment of the sections, and it is believed that, as a result of the relatively short time of exposure of the roots to C^{14}-adenine and the low concentration of the C^{14}-adenine used, the incorporation was primarily into RNA.

Roots were also treated with RNA-ase (1 mg/ml) while still attached to the bulb, beginning at 2:30 for between $^1/_2$ and 20 hours. They were then excised and given the active compounds for 3 hours, and autoradiographs were made in the usual way.

With the liquid-emulsion type autoradiograph, the site of activity is visualized by a series or track of silver grains, 4–12 in number. As the grains are spaced farther apart at the origin of the track than they are at the end, one end of the track can be

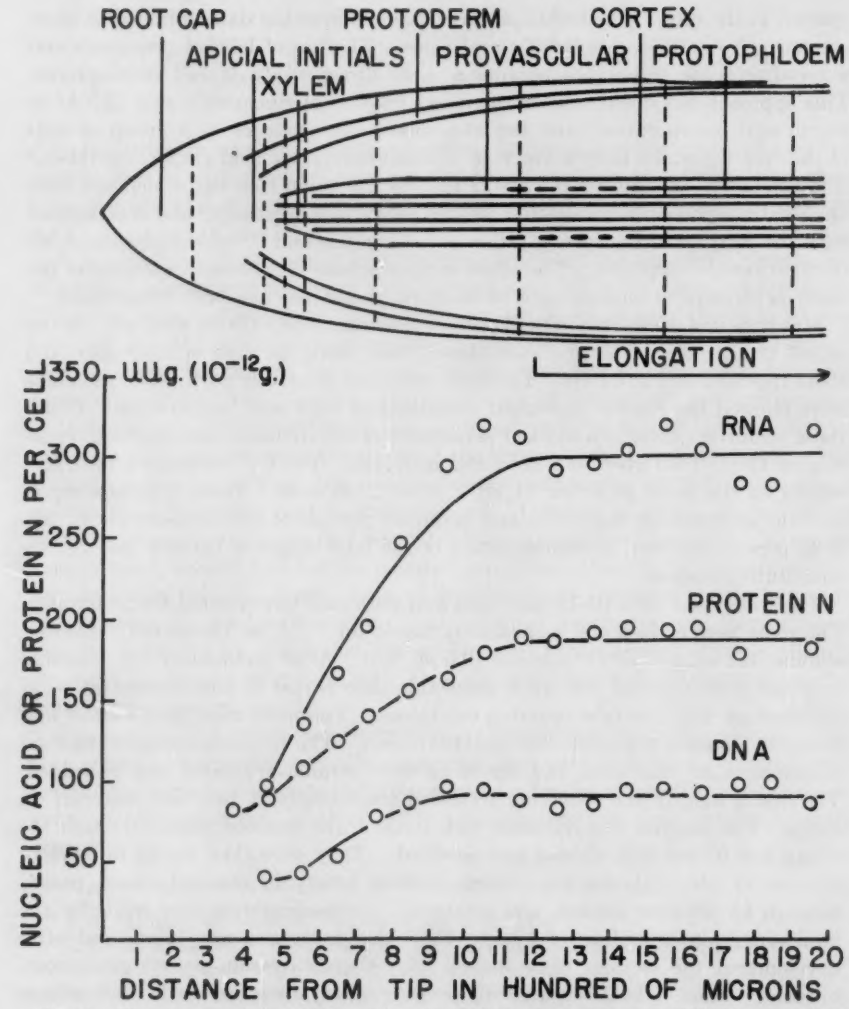

FIG. 1.—The amount of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein per cell of the onion root tip as a function of distance of section analyzed from the tip of the root. The diagram of the root is on the same scale as the abscissa of the graph.

identified from the other. The site of activity is assumed to be where the first grain of the track is located. The emitted electron may travel a short distance before ionizing a silver crystal, thus presenting a possible error in the counting. This error is believed negligible in most cases, being largest, however, in the smallest site scored, the nucleolus. The tracks were counted for the nucleolus, nucleus, and cytoplasm for the root cap, protoderm, cortex, and vascular tissues at the eight levels indicated by the broken lines on the diagram of the root in Figure 1. In the more basal sections the cells and nuclei of some of the tissues were larger than the

10-μ section counted. Correction factors, based on the actual size of the cells and nuclei, were used so that the data could be expressed per cell or per nucleus.

The protein, RNA, and DNA contents per cell of the first twenty 100-μ sections of the root were determined by the usual histochemical procedures.[4]

Morphology and Protein-Nucleic Acid Content of the Root.—The first 2 mm. of the onion root tip are represented diagrammatically in Figure 1. A detailed morphological analysis has been published and will only be summarized briefly here.[3] The first 400 μ of the root consist of the root cap, which, above this level, continues to surround the root for almost the entire 2 mm. The region of the apical initials is immediately basal to and surrounded by the root cap. The cells of this region appear either not to divide or to divide very slowly. The protoderm, cortex, and vascular tissues differentiate from the apical initials some 500 μ from the tip. In all cases, morphological differentiation involves differences in rate and extent of radial enlargement, there being but limited differences in cell length either within or between the various tissues. Elongation begins at approximately 1,100 μ from the tip, when radial enlargement is almost complete, and continues through the next several millimeters. The principal exception to this general pattern is the xylem. Cells of the xylem undergo both radial enlargement and elongation at the same time; elongation begins at 500 μ from the tip. The other vascular cells follow the generalized pattern.

Divisions occur in all tissues; the only non-dividing cells present are those of the extreme root cap and the apical initial group. The morphological diversity of cells in which divisions occur does not allow the generalization of a meristematic cell as a distinct cell type. Division appears to be a potentiality of cells of unlike morphology, not necessarily affecting the course of development of the dividing cell. Under the growth conditions used here, there is a marked periodicity of cell division, the maximum number occurring over noon and midnight. At these times, roughly 30 per cent of the cortical cells divide. There are 2–5 per cent of the cells dividing at the minimal periods.

The protein, RNA, and DNA content per cell is shown in Figure 1. The distribution of DNA per cell can be closely related to the number of cell divisions occurring in the various sections. The low value corresponds to the apical initial group, in which cell divisions are infrequent. Basal to this group of cells, the DNA per cell increases, apparently as a result of DNA doubling associated with cell division and polyploidy. The distributions of protein and RNA per cell are very similar and are correlated with cell development. The net increase in protein and RNA per cell occurs during the radial-enlargement stage, while the leveling-off per cell corresponds with the beginning of cell elongation.

Results: Incorporation of C^{14}-Phenylalanine and C^{14}-Adenine.—The results of the incorporation studies are presented in Figure 2 for the cortex and Figure 3 for the vascular tissue. Data for incorporation in the cytoplasm, the nucleus, and the nucleolus are given separately for each tissue, for each compound tested, and at two times—one of maximum cell division and one of minimum cell division.

Considering the data from the cortex first, the pattern of cytoplasmic incorporation of both substances can be divided into three stages: (1) low incorporation by the cells of the apical initial group, this incorporation increasing with distance from the tip; (2) a constant incorporation during radial enlargement, which ends

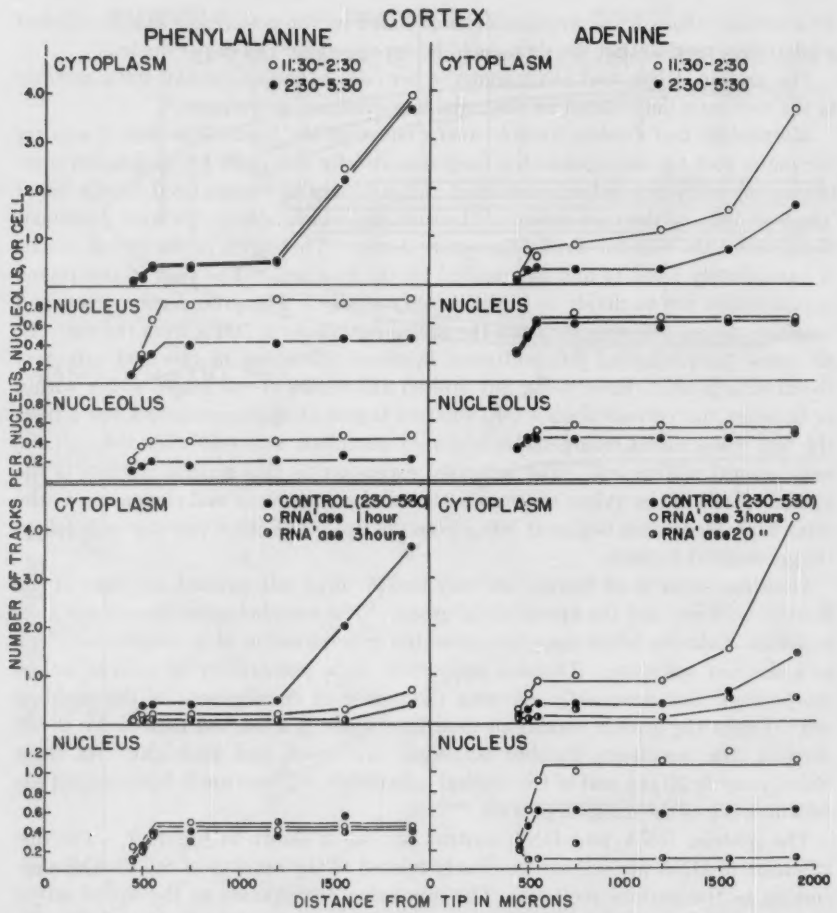

FIG. 2.—The incorporation of C^{14}-phenylalanine and C^{14}-adenine by the cytoplasm, nucleus, and nucleolus of the cells of the cortex between 11:30–2:30 P.M. and 2:30–5:30 P.M. The effect of ribonuclease treatment on the incorporation of these compounds is presented in the lower half of the graph.

with (3) a marked increase of incorporation with elongation. The incorporation by the nuclei and nucleoli is also low in the apical initials and increases to a constant amount some 550 μ from the tip; there is no change of incorporation of either compound with elongation.

When the data from the two time intervals, 11:30–2:30 and 2:30–5:30, are considered, a striking pattern becomes evident. With C^{14}-phenylalanine there is no difference in cytoplasmic incorporation between the two intervals, while there is a significant difference in nuclear and nucleolar incorporation, the earlier time being much higher than the later. With C^{14}-adenine there is a significantly higher incorporation by the cytoplasm during the 11:30–2:30 period compared with the 2:30–5:30 one, while there is no significant difference in nuclear and nucleolar

incorporation at the two times. The picture of incorporation of the two compounds by the protodermal cells is essentially the same as for the cortex.

The incorporation of the two compounds by the vascular tissue is markedly different from that in the cortex or protoderm. In the cells of the vascular tissue the cytoplasmic incorporation of C^{14}-phenylalanine increases steadily with distance from the tip until 1,150 μ, when there occurs a sharp increase in incorporation. There is no significant difference in incorporation during the two time periods. Nuclear incorporation of C^{14}-phenylalanine is similar to the cortex pattern, with the incorporation between 11:30 and 2:30 being twice that of the 2:30–5:30 period. Nucleolar incorporation is very low in the 2:30–5:30 period, being much higher in

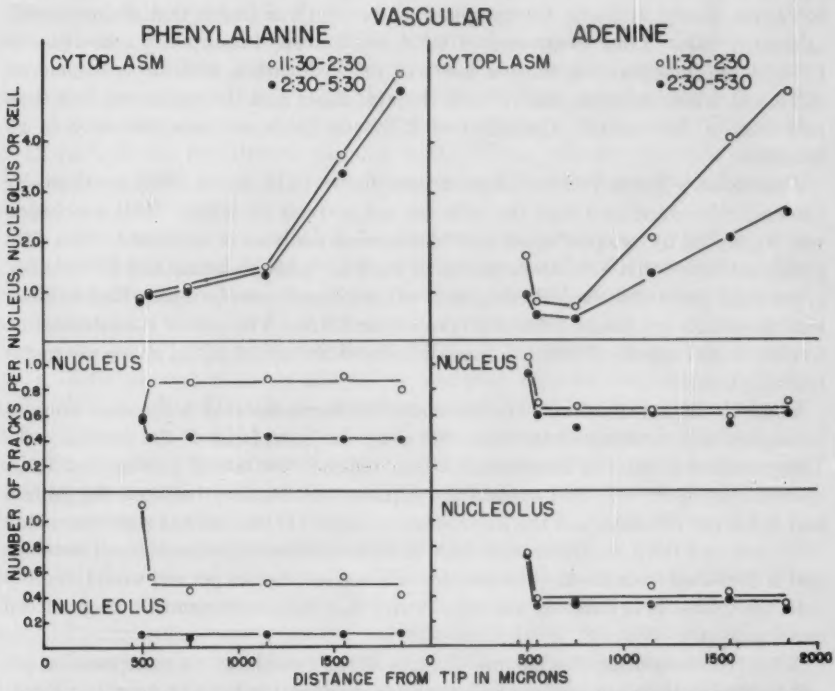

FIG. 3.—The incorporation of C^{14}-phenylalanine and C^{14}-adenine by the cytoplasm, nucleus, and nucleolus of the vascular cells between 11:30–2:30 P.M. and 2:30–5:30 P.M.

the 11:30–2:30 period, particularly so in the first section, where the earliest vascular cells differentiate. Incorporation of C^{14}-adenine by the cytoplasm, nuclei, and nucleoli of these first-formed vascular cells is also high. The amount of incorporation by the nuclei and nucleoli drops to a constant amount as the cells develop. The nuclear and nucleolar incorporation is not significantly different at the two time periods. Cytoplasmic incorporation of C^{14}-adenine also drops from the initial high but then increases markedly as the cells develop. As in the cortex, the amount of incorporation during the 11:30–2:30 period is much higher than during the 2:30–5:30 period.

The effect of RNA-ase treatment on the incorporation of C^{14}-adenine and C^{14}-phenylalanine by cells of the cortex is presented in Figure 1. Treatment of the root with RNA-ase has an almost immediate effect on the amount of incorporation of both compounds. RNA-ase treatment for 1 hour caused a decrease of 30 per cent in C^{14}-phenylalanine incorporation in cells 500–1,150 μ from the tip, and up to 80 per cent in more basal cells. Treatment for 3 hours resulted in a 60 per cent decrease in the early stages of development and close to a 90 per cent decrease in the later ones. Strikingly, RNA-ase treatment did not affect nuclear incorporation of C^{14}-phenylalanine.

The incorporation of C^{14}-adenine by the cells after short exposures to RNA-ase ($^1/_2$–3 hours) was not decreased but increased. After a 3-hour treatment of RNA-ase, incorporation by the cytoplasm and nuclei was double that of the control, untreated cells. Long treatments of RNA-ase, however, resulted in a decrease in C^{14}-adenine incorporation until a low was reached with a 20-hour treatment of RNA-ase, when incorporation of both the cytoplasm and the nuclei was less than half that of the control. The effect of RNA-ase treatment was the same in all tissues.

Discussion.—The apical initials have been shown to be low in DNA synthesis by Clowes,[2] who concluded that the cells are not actively dividing. This conclusion was supported by morphological and biochemical analyses of sections.[3] The data presented here—that the incorporation of both C^{14}-phenylalanine and C^{14}-adenine is low in all parts of the apical initial cells at both times—can be interpreted to mean that these cells synthesize little or no protein or RNA. The sum of the information to date would appear to indicate that the cells of the apical initial group are metabolically inactive.

Basal to the apical initials, the incorporation increases until a constant amount is reached and maintained for some 600 μ by the cytoplasm of the cortical cells. This constant amount of incorporation may indicate the rate of protein and RNA synthesis by these cells and explain the apparent discrepancy between the protein and RNA per cell data and the incorporation data. If the cell has a constant rate of protein and RNA synthesis with little or no breakdown, then, as the cell develops and is displaced from the tip, the amount of these substances per cell would steadily increase. This is apparently the case during the radial enlargement stage of cell development.

With cell elongation, this pattern changes in the cytoplasm, as incorporation per cell increases while amount per cell remains constant for both protein and RNA. This may be the result of rapid synthesis accompanied by rapid breakdown, which would not affect the total protein or RNA per cell, or a turnover or exchange phenomenon which would have the same end result. In either case there is a definite change in both protein and RNA metabolism associated with elongation.

This pattern of cytoplasmic incorporation is not changed with cell division in the case of C^{14}-phenylalanine and only slightly with C^{14}-adenine, although with the latter the amount of incorporation is increased. This increased incorporation of C^{14}-adenine with division but without a change in the pattern of incorporation with development lends support to the concept that division is the property of cells of unlike morphology and does not necessarily affect the course of development.

The pattern of incorporation of both compounds by the nuclei and nucleoli of the

cells differs most markedly from the cytoplasm when elongation begins. The incorporation by the nuclei and nucleoli is low in the apical initials, increases as the cells begin active development to a constant amount, which is maintained for the entire length of the root analyzed here. Moreover, there is no significant difference in the amount of C^{14}-adenine incorporated at the two times, while the amount of C^{14}-phenylalanine incorporated between 11:30–2:30 is much greater than between 2:30–5:30. This is the exact opposite of the data from the cytoplasm. The C^{14}-phenylalanine incorporation by nucleoli during the 2:30–5:30 interval is very low. The data suggest the possibility that protein synthesis associated with the nucleolus occurs in the later stages of division and that there is little associated with the nucleolus of the non-dividing cell.

This general interpretation of the results is apparently also true for the vascular cells. These cells are undergoing both radial enlargement and elongation at all times, and the incorporation data from the cytoplasm can be interpreted as a combination of these two patterns. The unique feature of the vascular cells is the very high rate of incorporation of C^{14}-phenylalanine by the nucleolus and C^{14}-adenine by all parts of the first-formed vascular cells. These cells are unusually large in cross-sectional area, and it is possible that this high incorporation is a reflection of this phase of their differentiation.

The data obtained from the RNA-ase-treated roots indicates a very definite relation between RNA and C^{14}-phenylalanine incorporation by the cytoplasm. The data presented here confirm the observations of Brachet[1] on the incorporation of both compounds after RNA-ase treatment of root tips and lends support to the work of Webster.[7] The lack of effect of RNA-ase treatment on nuclear incorporation of C^{14}-phenylalanine is interesting and may support the work of Allfrey,[8] which indicates that DNA is associated with amino acid incorporation in the nucleus. The effect of RNA-ase on C^{14}-adenine incorporation can be divided into two periods: $^1/_2$–3 hours in RNA-ase results in increased adenine incorporation, while longer periods result in decreased incorporation. No clear explanation for this phenomenon is available at present.

Summary.—The incorporation of C^{14}-phenylalanine and C^{14}-adenine was followed in cells of the root tip of *Allium cepa* by autoradiographic techniques. The first 2 mm. of the root were used and contain, besides the root cap, the apical initials, the radial-enlargement, and early elongation stages of cell development. The apical initials have a very low incorporation of both compounds, which is interpreted to mean little or no protein or RNA synthesis. The cells in radial enlargement have a steady rate of incorporation which appears to be an indication of steady protein and RNA synthesis with time, which results in a net increase in both as the cells are displaced from the tip. With elongation, cytoplasmic incorporation increases greatly, while the RNA and protein content of the cells remain constant, indicating either a marked increase of synthesis with an equally marked increase in breakdown or an exchange phenomenon associated with elongation. There is no change in nuclear or nucleolar incorporation of either compound with elongation. Cytoplasmic incorporation of C^{14}-adenine is higher when divisions occur, while the incorporation of C^{14}-phenylalanine is unaffected by cell division. Nuclear and nucleolar incorporation of C^{14}-phenylalanine is much higher during division, while C^{14}-adenine incorporation is not affected. Treatment of the roots with RNA-ase

before the incorporation period resulted in a marked effect on C^{14}-phenylalanine incorporation by the cytoplasm, while not affecting nuclear incorporation. The incorporation of C^{14}-adenine was increased in roots with short treatments of RNA-ase and decreased in roots given long treatments.

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*THE OPTICAL ROTATORY DISPERSION OF THE α-HELIX**

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In earlier articles[1, 2] we have applied the polarizability theory of optical activity[3] to determine for the sodium D line the specific rotation due to the helical conformation alone for an infinitely long right-handed polypeptide α-helix. Recent experiments[4–7] indicate that the rotatory dispersions of poly-γ-benzyl-L-glutamate and poly-L-glutamic acid in the α-helical conformation are anomalous. Moffitt[8, 9] has proposed that this anomalous dispersion is due to special terms which arise from the $N \rightarrow V_1$ and $N \rightarrow V_2$ electronic transitions in the chromophores of the NCO groups of the polypeptide residues which make up the helical chain. The relationship between Moffitt's calculations of these special terms and the polarizability theory is discussed elsewhere.[10]

The model of the α-helix used in our original calculations,[1] where the total polarizability of each peptide residue is uniformly distributed with an optical axis of symmetry parallel to the direction of the helix, does not account for the observed dispersion associated with the two NCO electronic transitions considered by Moffitt. While a model assuming uniform group polarizability may be approximately valid on the long-wave-length side, it must necessarily fail in a region of anomalous dispersion arising from electronic transitions in a particular group, unless all groups possess identical spectra. This model, which ignores the structural details of the NCO group, is therefore inadequate to describe observed behavior in the immediate neighborhood of the special NCO frequencies where their contribution dominates through anomalous dispersion.

In this article we make a synthesis of Moffitt's model[9] and a modified version of our original model[1] and apply the polarizability theory to calculate its rotatory dis-

persion. The effective molecular rotation $[m']$ is related to the specific rotation $[\alpha]$ and the molecular rotation $[M]$ by

$$[m'] = \frac{3}{n_\nu^2 + 2}\frac{[\alpha]M}{100} = \frac{3}{n_\nu^2 + 2}[M], \tag{1}$$

where M is the residue molecular weight and n is the refractive index of the solution for incident plane-polarized light of frequency ν. The advantage of using $[m']$ is that it is dependent only on the helical structure and not on the solvent or the side groups. According to the polarizability theory, the effective molecular rotation $[m']$ is related to the molecular rotatory parameter $g^{(0)}$ by

$$[m'] = 280.0\ \pi^2 N_0 c^2 \nu^2 g^{(0)}, \tag{2}$$

where N_0 is Avogadro's number and c is the speed of light.

In the analysis to follow, we designate equations in Kirkwood's original article[3] by equation (K). Substitution of equation (K28) in equation (K24) with a rearrangement of terms leads at once to the following general expression for $g^{(0)}$:

$$g^{(0)} = \frac{2}{3h^2}\sum_{n_i, n_k'}\frac{A_{n_i,\ n_k}}{(\nu_{n_{i0}}{}^2 - \nu^2)\ (\nu_{n_{k0}}{}^2 - \nu^2)},$$

$$A_{n_i\ n_k'} = \sum_{i<k=1}^{N} \nu_{n_{i0}}\nu_{n_{k0}}(V_{ik})_{n_{i0};\ n_{k0}}\boldsymbol{R}_{ik}\cdot(\mu_{n_{i0}}{}^{(i)} \times \mu_{n_{k0}}{}^{(k)}), \tag{3}$$

$$\boldsymbol{R}_{ik} = \boldsymbol{R}_k - \boldsymbol{R}_i,$$

for a molecule consisting of N groups, with unperturbed absorption frequencies ν_{n_i0}, in a dissymmetric conformation. The quantities $\mu_{n_{i0}}{}^{(i)}$ and $(V_{ik})_{n_{i0};\ n_{k0}}$ are the appropriate matrix elements of the electric dipole moments of the groups and of the interaction Hamiltonians V_{ik} of pairs of groups i and k separated by the vector distances $\boldsymbol{R}_{ik}$, and h is Planck's constant.

Equation (3) represents the quantum-mechanical form of the Born coupled oscillator theory of optical activity. In the original theory of Kirkwood, equation (3) was bypassed by making a simultaneous substitution of equations (K28) and (K25) in equation (K24). Equation (K25) involved the explicit use of dipole-dipole coupling to represent V_{ik},

$$V_{ik} = \mu^{(i)}\cdot\boldsymbol{T}_{ik}\cdot\mu^{(k)},$$

$$T_{ik} = \frac{1}{R_{ik}{}^3}\left[1 - \frac{3\boldsymbol{R}_{ik}\boldsymbol{R}_{ik}}{R_{ik}{}^2}\right]. \tag{4}$$

These steps lead to the central result of the polarizability theory,

$$g^{(0)} = \frac{1}{6}\sum_{i<k=1}^{N}\sum_{r,s=1}^{3}\alpha_{rr}{}^{(i)}\alpha_{ss}{}^{(k)}\ (\boldsymbol{b}_r{}^{(i)}\cdot\boldsymbol{T}_{ik}\cdot\boldsymbol{b}_s{}^{(k)})\ \times$$

$$\boldsymbol{R}_{ik}\cdot(\boldsymbol{b}_r{}^{(i)} \times \boldsymbol{b}_s{}^{(k)}), \tag{5}$$

where the vectors $\boldsymbol{b}_r{}^{(i)}$ are the principal axes and the quantities $\alpha_{rr}{}^{(i)}$ are the components of the polarizability tensor $\alpha^{(i)}$ of group i. Equation (5) is identical with

equation (K33) except for a correction in sign which was made in a subsequent article[11] and reduces to equation (K35) with corrected sign when all group polarizability tensors possess axes of symmetry. Although Kirkwood derived equation (3) only for the nondegenerate case, Moffitt, Fitts, and Kirkwood[10] have shown that equation (3) is also valid for the degenerate case, e.g., for polyamino acids.

We now apply equation (3) to the calculation of the optical rotatory dispersion of the α-helix, after explicitly introducing dipole-dipole interaction between pairs of groups, as expressed in equation (4). The centers of mass of the nitrogen and carbon atoms which form the main structural chain are placed on right-handed cylindrical helices. The equations of the helix passing through the nitrogen atoms are $x = a_1 \cos\theta$, $y = a_1 \sin\theta$, $z = b\,\theta$; through the carbonyl carbon atoms, $x = a_2 \cos\theta$, $y = a_2 \sin\theta$, $z = b\,\theta$; and through the asymmetric carbon atoms, $x = a_3 \cos\theta$, $y = a_3 \sin\theta$, $z = b\,\theta$. The values of the radii a_1, a_2, a_3, of the pitch b, and of the angle between the ith and the kth atoms, $\theta_{ik} = \theta_k - \theta_i$, for an 18-residue 5-turn α-helix are given by Pauling and Corey.[12]

The molecular rotatory parameter $g^{(0)}$ is split into the sum of three parts, $g_1{}^{(0)}$, $g_2{}^{(0)}$, and $g_3{}^{(0)}$. The first part, $g_1{}^{(0)}$, which is called the special term, arises from the interactions of the $N \rightarrow V_1$ and $N \rightarrow V_2$ electronic transitions in the NCO groups with one another. When Pauling and Corey's co-ordinates are used, the unit vectors in the directions of the $N \rightarrow V_1$ and $N \rightarrow V_2$ transitions are

$$\boldsymbol{b}_i = -\boldsymbol{i}\cos\phi(\cos 22^\circ \sin\theta_i + \sin 22^\circ \cos\theta_i) + \boldsymbol{j}\cos\phi(\cos 22^\circ \cos\theta_i - \sin 22^\circ \sin\theta_i) + \boldsymbol{k}\sin\phi, \quad (6)$$

where ϕ is the angle between the direction of polarization of the transition and its projection on the $(x - y)$ plane. The constants $A_{ni,nk}$ in equation (3) become

$$A_{jj} = \sum_{i<k=1}^{N} \nu_{j0}{}^2 \mu_{j0}{}^{(i)2} \mu_{j0}{}^{(k)2} (\boldsymbol{b}_j{}^{(i)} \cdot \boldsymbol{T}_{ik} \cdot \boldsymbol{b}_j{}^{(k)} \times [-4a_1 \sin\phi \cos\phi \cos 22^\circ \sin^2\frac{\theta_{ik}}{2} + b\cos^2\phi\,\theta_{ik}\sin\theta_{ik}], \quad (7)$$

$$A_{jm} = \sum_{i<k=1}^{N} \nu_{j0}\nu_{m0}\mu_{j0}{}^{(i)2}\mu_{m0}{}^{(k)2}(\boldsymbol{b}_j{}^{(i)} \cdot \boldsymbol{T}_{ik} \cdot \boldsymbol{b}_m{}^{(k)} \times [a_1 \sin 22^\circ \sin\theta_{ik} - 2\,a_1 \cos 2\phi \cos 22^\circ \sin^2\frac{\theta_{ik}}{2} - b\sin\phi\cos\phi\,\theta_{ik}\sin\theta_{ik}],$$

$$j = 1, 2; \qquad m = 1, 2, \neq j.$$

These constants A_{jj} and A_{jm} represent the interactions of the $N \rightarrow V_j$ transition in one residue with the $N \rightarrow V_j$ and $N \rightarrow V_m$ transitions, respectively, in the other residues.

The Drude term, $g_2{}^{(0)}$, arises from the interactions of these two special NCO electronic transitions with other electronic transitions in the helical chain. The absorption frequencies of electronic transitions other than these two special ones in the NCO groups lie well outside the visible and near ultraviolet regions. Therefore, the expression $(\nu_{n_{k0}}{}^2 - \nu^2)$ associated with each electronic transition of frequency $\nu_{n_{k0}}$ is effectively a constant in the visible absorption region. The only

frequency dependence remaining in $g_2^{(0)}$ is $1/(\nu_{n_{f0}}^2 - \nu^2)$, where $\nu_{n_{f0}}$ is the absorption frequency of either the $N \rightarrow V_1$ or the $N \rightarrow V_2$ transition in the NCO group.

The third part, $g_3^{(0)}$, of the molecular rotatory parameter arises from the interactions of electronic transitions other than the two special NCO ones previously considered. Since the absorption frequencies of these transitions are far removed from the visible, the term $g_3^{(0)}$ is effectively a constant.

The orientations of the transition moments of the $N \rightarrow V_1$ and $N \rightarrow V_2$ electronic transitions in amides have been determined by Peterson and Simpson.[13] When their results are applied to the Pauling and Corey model of the α-helix, the angle ϕ in equation (7) becomes $-38°$ for the $N \rightarrow V_1$ electronic transition and $52°$ for the $N \rightarrow V_2$ transition. The dipole moments in equation (7) can be calculated by the relation

$$\mu_{n_{f0}}^2 = \frac{3e^2h}{8\pi^2m} \frac{f_{n_{f0}}}{\nu_{n_{f0}}}, \tag{8}$$

where e and m are the electronic charge and mass, respectively, and $f_{n_{f0}}$ is the oscillator strength of the transition. On the basis of available measurements on

TABLE 1

CALCULATED OPTICAL ROTATORY DISPERSION OF THE α-HELICAL CONFIGURATION

λ (mμ)	$[m_1']$	$[m_{2+}']$	$[m_{2-}']$	$[m_3']$	$[m']$ Theoretical	$[m']$ Experimental
750	32°	277°	−268°	20°	61°	55° ± 4°
700	37	320	−311	23	69	61 ± 3
650	44	374	−365	27	80	72 ± 3
600	53	443	−436	31	91	86 ± 3
589	56	461	−453	32	96	90 ± 3
550	66	534	−529	37	108	104 ± 3
500	84	656	−657	45	128	125 ± 4
450	112	829	−843	56	154	153 ± 5
425	131	944	−969	62	168	171 ± 7
400	158	1,084	−1,128	70	184	189 ± 8
375	193	1,262	−1,334	80	201	209 ± 10
350	243	1,488	−1,607	92	216	227 ± 13

amides,[14, 15] Moffitt[9] has inferred that the $N \rightarrow V_1$ NCO electronic transition lies at 1850 A, with an oscillator strength of 0.237 and the $N \rightarrow V_2$ transition lies at 1480 A with an oscillator strength of 0.209. The contributions of the special term $g_i^{(0)}$ at various wave lengths to the total effective molecular rotation $[m']$ are listed in Table 1 under the heading $[m_1']$.

The contribution to the mean polarizability α of the NCO group from these two special electronic transitions for incident light of sodium D wave length is

$$\alpha = \frac{e^2}{4\pi^2m}\left(\frac{f_1}{\nu_{10}^2 - \nu_D^2} + \frac{f_2}{\nu_{20}^2 - \nu_D^2}\right) = 0.992 \text{ A}^3. \tag{9}$$

The total mean polarizability of the NCO group for the sodium D line as determined from atomic refractions[1] is 3.24 A^3. The difference between these two values is attributed to the other electronic transitions in the NCO group and is distributed equally between the nitrogen and carbon atoms. The principal axis of the polarizability tensor of these latter contributions is taken to be tangent to the helix passing

through the center of mass of that particular atom. The mean polarizability of the CH group is centered at the asymmetric carbon atom and is similarly directed.

This model differs from our original one[1] in that the polarizability due to the two special NCO electronic transitions is segregated and the remaining polarizability is centered at the nitrogen and carbon atoms of the main helical chain rather than being continuously distributed. We also take into account the different distances of the various atoms from the z-axis rather than assume a common average distance.

In the calculation of $g_2^{(0)}$ from equations (3) and (4), the dipole moments associated with the two special NCO transitions are given by equation (8), and those associated with the other electronic transitions are related to the mean polarizability α by

$$\alpha = \frac{2}{3h} \sum_k{}' \frac{\nu_{k0}\mu_{k0}^2}{\nu_{k0}^2 - \nu^2}. \tag{10}$$

The Drude term $g_2^{(0)}$ is divided into two contributions: that involving the $N \rightarrow V_1$ NCO transition and that involving the $N \rightarrow V_2$ transition. The contributions to $[m']$ of the former at various wave lengths are listed in Table 1 under $[m_2{}^-{}']$ and of the latter under $[m_2{}^+{}']$.

The constant term is calculated in the same manner as in our previous article[1] except that the terms are summed rather than integrated. The contribution of the constant term is listed in Table 1 under $[m_3']$.

In these calculations we assign a conventional value of $^1/_3$ to the anisotropies β of the polarizabilities not associated with the two special NCO electronic transitions. Although the validity of this assignment has been questioned,[9] the anisotropy ratios β for most non-aromatic molecular groups with cylindrical symmetry are observed[3] to be approximately $^1/_3$. Since in this model of the α-helix the polarizability due to the two special NCO transitions is segregated and the remaining polarizability is centered at specific atoms, we believe that the assignment of about $^1/_3$ for β is reasonable and valid.

In calculating the constants A_{n_i,n_k}, which occur in the special, Drude, and constant terms, we sum the interactions of each point dipole with all the other point dipoles on the helical chain until the distance between pairs is sufficiently large to make their contribution negligible; usually about twelve residues on each side are sufficient. In order to remove the contributions of the asymmetric centers on the helical chain and thereby obtain the contribution of the helical structure alone, we neglect interactions between point dipoles on adjacent atoms. These terms which are omitted do not accurately represent the contributions of the asymmetric centers in the random-coil form of the polypeptide, but we believe that we are more justified in omitting than in keeping them. Of course, we also omit interactions involving the side groups.

The total effective molecular rotation is given by

$$[m'] = [m_1'] + [m_{2+}'] + [m_{2-}'] + [m_3'] \tag{11}$$

and is compared in Table 1 with the experimental rotatory dispersion of Doty and Lundberg.[7] In Table 2 we present theoretical and experimental values of $[m']\lambda^2$

for various wave lengths. Here we can see more clearly the nature of the anomalous dispersion. We observe that the theoretical calculations agree well with the experimental values and that the anomalous rotatory dispersion is the result of a large negative Drude term which is prevented from completely dominating the rotatory power by a positive Drude term and a positive special term.

Doty and Lundberg[7] have fitted their experimental data with an equation suggested by Moffitt.[8] This equation consists of a single Drude term and a single special term, both with the same frequency of absorption. When the parameters of this empirical equation are evaluated for the experimental dispersion, the Drude term is positive and the special term is negative. The special term of Moffitt's equation does not represent the same contributions to the optical rotation as our special term. Our special term, in fact, has the opposite sign to that determined for the empirical equation. Moreover, our calculations show that the representation of the experimental data by an empirical equation of the Moffitt form is not unique.

Elliott, Hanby, and Malcolm[16] have measured the contribution of the helical structure of poly-L-leucine to the optical rotation for the sodium D line and found that $[m']_D$ is $+97°$. Our calculated result for a right-handed α-helix agrees well with this value.

TABLE 2

CALCULATED ANOMALOUS DISPERSION OF THE α-HELIX

λ ($m\mu$)	$\lambda^2[m_1']$ $\times 10^9$	$\lambda^2[m_{2+}']$ $\times 10^9$	$\lambda^2[m_{2-}']$ $\times 10^9$	$\lambda^2[m_4']$ $\times 10^9$	$\lambda^2[m'] \times 10^9$ Theoretical	Experimental
750	179	1,558	−1,510	113	340	309
700	182	1,568	−1,524	113	339	299
650	187	1,580	−1,544	113	336	304
600	192	1,595	−1,568	113	332	310
589	194	1,598	−1,573	113	332	312
550	200	1,615	−1,600	113	328	315
500	209	1,641	−1,643	113	320	313
450	226	1,679	−1,707	113	311	310
425	237	1,704	−1,750	113	304	309
400	252	1,735	−1,804	113	296	302
375	271	1,774	−1,875	113	283	294
350	298	1,823	−1,968	113	266	278

By a method which avoids the use of the dipole-dipole coupling approximation, Moffitt[9] has calculated the matrix elements for the interaction Hamiltonians which occur in the coefficients A_{11} and A_{22} of the special term $g_1^{(0)}$. With one exception, these matrix elements agree in sign and, within ± 50 per cent, in magnitude with the dipole-dipole terms used in our calculation of A_{11} and A_{22}. We believe, therefore, that the approximation of dipole-dipole coupling between residues leads to the correct sign for these terms.

Although we are fortunate in obtaining such a good comparison with experiment, we do not believe that the approximations in our model and the use of dipole-dipole interactions at short distances warrant such good agreement. The polarizability theory can, however, predict the trend of the rotatory dispersion and indicates that the special term is positive for a right-handed helix. Our original conclusion,[2] that the α-helix for naturally occurring poly-L-peptides is right-handed, remains valid and agrees with the tentative assignment of Elliott and Malcolm[17] based on analysis of X-ray diffraction patterns.

Our original model of the α-helix[1] is certainly no more than a conventional facsimile of the actual polypeptide chain. If Moffitt had confined the criticism con-

tained in his first three comments[9] to an expression of skepticism concerning the numerical precision of estimates of optical activity based upon our model, we would acknowledge agreement with him. However, consideration of a right-handed helical array of point-polarizable groups with centers coincident with the carbon and nitrogen atoms of the chain and with directions of polarizability either parallel or perpendicular to the helical direction leaves our conclusion unchanged.

We have attributed[2] the algebraic decrease in the specific rotation $\Delta[\alpha]_D$ of poly-γ-benzyl-L-glutamate, observed by Yang and Doty,[6] in solvents destroying the helical structure principally to the annihilation of a positive contribution to the rotation from a right-handed α-helix. We have always been completely aware of the possibility that a part of this change may be due to a change in the intrinsic rotations of the side chains produced by an altered intramolecular environment. However, this change in intrinsic rotations of the side chains is difficult to account for quantitatively. We simply compared our theoretical calculation with the only experimental data[4] available at that time. Subsequently, Doty and Lundberg[7] succeeded in determining the optical rotation of poly-γ-benzyl-L-glutamate due to the α-helical structure alone. Their results indicate that the changes in intrinsic rotations of the side groups are of considerably smaller magnitude than $\Delta[\alpha]_D$ and do not affect our assignment of a right-handed helical configuration.

In conclusion, we wish to thank Professor P. Doty for providing us with the experimental optical rotatory dispersion for the helical configuration of poly-γ-benzyl-L-glutamate prior to publication and for estimating the experimental error in these data. We also wish to thank Professor W. Moffitt for giving us his calculated matrix elements for the interaction Hamiltonians for the two special NCO electronic transitions.

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*INVARIABLE GENETIC CHANGE IN MAIZE PLANTS HETEROZYGOUS FOR MARBLED ALEURONE**

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A previous study[1] has shown that the R^r male gametes formed by R^rR^r and R^rR^{st} maize plants are genetically different. (R^r = self-colored aleurone; R^{st} = stippled aleurone; r^r = colorless aleurone). The aleurone is darkly mottled following r^rr^r ♀ × R^rR^r ♂ testcrosses, whereas all the $R^rr^rr^r$ kernels resulting from r^rr^r ♀ × R^rR^{st} ♂ matings are weakly pigmented. The altered form of R^r arising in R^rR^{st} heterozygotes, originally termed R' (now designated $R^{r:st}$), is transmitted in this form by the $R^{r:st}r$ testcross offspring. $R^{r:st}$, however, regularly reverts partially toward standard R^r in $R^{r:st}R^{r:st}$ homozygotes extracted from R^rR^{st} individuals by selfing. No change in the determinative action of the R^{st} male gametes from R^rR^{st} plants was observed, although the possibility that an inconspicuous alteration in phenotypic effect occurs has not been excluded.

The investigations now to be reported reveal that genetic change of this kind affecting the R^r phenotype is not unique in R^rR^{st} heterozygotes but occurs also when another allele, marbled (R^{mb}) is substituted for stippled. The changes in R^r arising in R^rR^{mb} and R^rR^{st} plants are not the same, but the two cases are parallel.

Additional observations on stippled made since a preliminary account of the action of this allele was published will be briefly mentioned here because of their relevance to the effect of marbled also. (1) The inherited shift in R^r phenotype arising in R^rR^{st} plants is not due to a cytoplasmic factor. R^rr^r plants, in which R^r was derived from the standard R^rR^r strain and r^r from an $R^{st}r^r$ heterozygote, used in testcrosses on r^gr^g ♀ ♀ (colorless aleurone, green plant), give $R^rr^gr^g$ kernels as darkly mottled as those resulting from r^gr^g ♀ × standard R^rR^r ♂ matings. (2) The earlier uncertainty concerning the amount of reversion toward the standard level of R^r pigmentation of $R^{r:st}$ in $R^{r:st}R^{r:st}$ homozygotes derived from R^rR^{st} by selfing has now been resolved. The order, in terms of pigment-producing action of the three forms of R in question, in $R\ r\ r$ aleurone cells, is $R^{r:st}$ < reverted $R^{r:st}$ < standard R^r. Thus, reversion of $R^{r:st}$ toward standard R^r regularly occurs in $R^{r:st}R^{r:st}$ homozygotes, but it is partial and not complete, as was first thought might be the case.[1] Standard R^r and reverted $R^{r:st}$ were found to give overlapping phenotypes on the inbred 4Co63 r^rr^r stock originally used as the ♀ ♀ tester but have proved readily distinguishable in matings with another inbred colorless aleurone line, W23. (3) The change from R^r to $R^{r:st}$ regularly occurring in R^rR^{st} heterozygotes is now known to be transmitted by the eggs as well as the sperm, which alone were effectively sampled in the initial experiments. It remains to be determined, however, whether the degree of change in R^r is the same in both cases. (4) Two genes for colorless aleurone, r^r ("red" plant) and r^g ("green" plant), of different origin than the R^r gene in question, do not alter the pigment-producing action of R^r in the respective R^rr^r and R^rr^g heterozygotes.

With this background, the experiments showing that action of the marbled allele in R^r heterozygotes is similar to that of stippled may now be considered.

MATERIALS AND METHODS

Marbled (R^{mb}), like stippled, conditions a spotting pattern for anthocyanin pigmentation in the aleurone, the outer layer of endosperm cells. The two patterns, however, are distinct. Marbled kernels, as the name suggests, are characterized by few, relatively large, irregularly shaped patches of solid color on a colorless background, whereas stippled aleurone is finely and densely dotted. The two alleles have not been observed to mutate to each other. Occasional homozygous marbled kernels are either self-colored or colorless. We have not determined how frequently these extreme deviates represent germinally transmissible mutations. By analogy with stippled, which gives self-colored offspring at the rate of about 3 per 10^3 individuals in this stock, it is expected that some of the infrequently occurring fully pigmented kernels on marbled ears will prove to be heritable also. Homozygous marbled plants do not form detectable amounts of anthocyanin in the roots or first internode above the cotyledon (when these tissues are exposed to light) or in the anther, although the coleoptiles may be slightly pigmented.

Where homozygous marbled has been used as the pollen parent in outcrosses to colorless aleurone, the result has been either that the resulting kernels are colorless or show small pigmented areas in some 1 per cent of the cases. $R^{mb}R^{mb}r$ kernels, on the other hand, usually are spotted.

The R^r allele, used in the present experiments, was the same as that employed in the previous study[1] of stippled. It conditions self-colored aleurone in $R^rR^rR^r$ and R^rR^rr kernels, and in single dose (R^rrr) gives dark mottling. This allele has been found to be comparable in stability to others of the R^r class studied by Stadler[2] in terms of mutation to r^r (colorless aleurone, red plant) and R^g (colored aleurone, green plant). The R^{mb} and R^r stocks had been made nearly isogenic with a highly inbred commercial strain designated W22. The colorless aleurone parent employed in most of the testcrosses was a second inbred line of Wisconsin origin, known locally as W23, but also as CC6. W23 is homozygous c and r^g, and thus forms no anthocyanin in either endosperm or plant. R^rr^g and $R^{mb}r^g$ seedlings arising from W23 $r^g \times 22$ R^rR^{mb} testcrosses are readily separable on the basis of pigmentation in root and first internode above the cotyledon. This criterion for verifying the particular colored allele present still serves when R^r is in the modified forms arising in R^rR^{st} or R^rR^{mb} heterozygotes.

Pollen from R^rR^r, R^rR^{mb}, and $R^{mb}R^{mb}$ sibs from selfed W22 R^rR^{mb} plants was applied to the W23 r^g strain. Composition of the staminate parents used in these testcrosses was verified by kernel phenotypes on the selfed ears. Some additional testcrosses were made in which the 4Co63 r^rr^r inbred line was substituted for W23, but the resulting kernels were not scored in detail when it was observed that the aleurone phenotypes paralleled those in the W23 series. Pollen from a standard culture of W22 R^rR^r into which neither R^{mb} nor R^{st} had been introduced was applied to W23 r^gr^g and 4Co63 r^rr^r plants. The resulting seeds served as the control for R^r pigmentation.

Pigmentation of the seeds obtained from the testcrosses was measured by the method previously applied by Brink.[1] A predetermined area on the abgerminal

side of each kernel was scanned under a dissecting microscope fitted with a 20 × 20 reticule covering about 12 mm.², at 27 × magnification. The sum of the totally or partially pigmented squares thus defined was divided by 4, and the quotient was then taken as the color index for the kernel. The scoring was done on a random sample of the more or less uniformly shaped seeds from the middle portion of the ear. Identification of the two classes of kernels resulting from the W23 $r^g r^g$ × W22 $R^r R^{mb}$ matings was verified retroactively on the basis of color in the root and first internode of seedlings grown from them.

This scoring method is objective, and served to resolve the genotypes under study. It overweights, however, the color scores for kernels in which the pigmented areas are scattered rather than continuous or close together, since a reticule square containing only two or three pigmented aleurone cells is given the same value as one which is solidly colored or densely spotted. This qualification of the scoring method is relevant to the comparisons between certain kernel classes made later in this report.

EXPERIMENTAL RESULTS

The distributions in terms of aleurone color index of the testcross kernels are assembled in Table 1. Each array represents the kernel sample from a single ear. The results are illustrated in the form of histograms in Figure 1.

The Control Mating, $r^g r^g$ ♀ × *Standard* $R^r R^r$ ♂.—The results of the matings in which pollen from the standard W22 $R^r R^r$ stock was applied to W23 $r^g r^g$ ear shoots are entered as the first group in Table 1 (lines 1–4). The modal color index for each of the four single-ear families is 98, the highest value on the scale. Approximately two-thirds of the kernels are in the modal class in each case, and most of the remainder fall in nearby classes. It is evident that this is a heavily pigmented group of kernels.

The $R^r r^g r^g$ *Kernels from the* $r^g r^g$ ♀ × $R^r R^{mb}$ ♂ *Mating.*—All the kernels from this mating were first scored for aleurone color by the method described earlier and then were separated into the two genotypic classes represented, $R^r r^g r^g$ and $R^{mb} r^g r^g$, by the seedling pigmentation test. Group 2 in Table 1 includes the $R^r r^g r^g$ kernels only. It is obvious from the distributions that, in general, this class of kernels is weakly pigmented. Five single-ear families are represented in the table, and in four of them the modal color index is 3, the lowest class value. The mode in the case of the fifth family (line 8) is ill defined but lies in the same region. The greater dispersion for color index of the group 2 kernels relative to that for group 1 may be real, although the difference in family size is a factor also. The group 2 distributions overlap only slightly with those of group 1. It is evident that the aleurone color-producing potential of the R^r male gametes, as a group, formed by $R^r R^{mb}$ plants is much lower than that of their counterparts from the $R^r R^r$ standard strain.

The modified form of R^r arising in $R^r R^{mb}$ heterozygotes will be referred to subsequently as $R^{r:mb}$.

Testcrosses Involving Extracted $R^{r:mb} R^{r:mb}$ *Plants.*—The R male gametes formed by $R^{r:mb} R^{r:mb}$ individuals derived from selfed $R^r R^{mb}$ plants have a much higher pigment-producing potential, on the average, than those from $R^r R^{mb}$ sibs, as the group 3 data in Table 1 show. About four-fifths of the kernels in group 3 gave color indexes of 78 or above, a range into which the $R^{r:mb} r^g r^g$ kernels from the

R^rR^{mb}♂ testcross extends in but a single case. There is overlap in the two sets of distributions in the middle of the scale only, and the amount is relatively small. Thus the striking reduction in pigment-producing potential of R^r which occurs in R^rR^{mb} heterozygotes is subject to a pronounced reversion in extracted $R^{r:mb}R^{r:mb}$ homozygotes.

Comparison of the distributions in group 3 with those in group 1 (testcrosses of standard R^rR^r♂♂) discloses that the reversion in the extracted $R^{r:mb}R^{r:mb}$ homozygotes toward the level of standard R^r is not complete. The data as presented in the table provide clear evidence in support of this conclusion. The case for

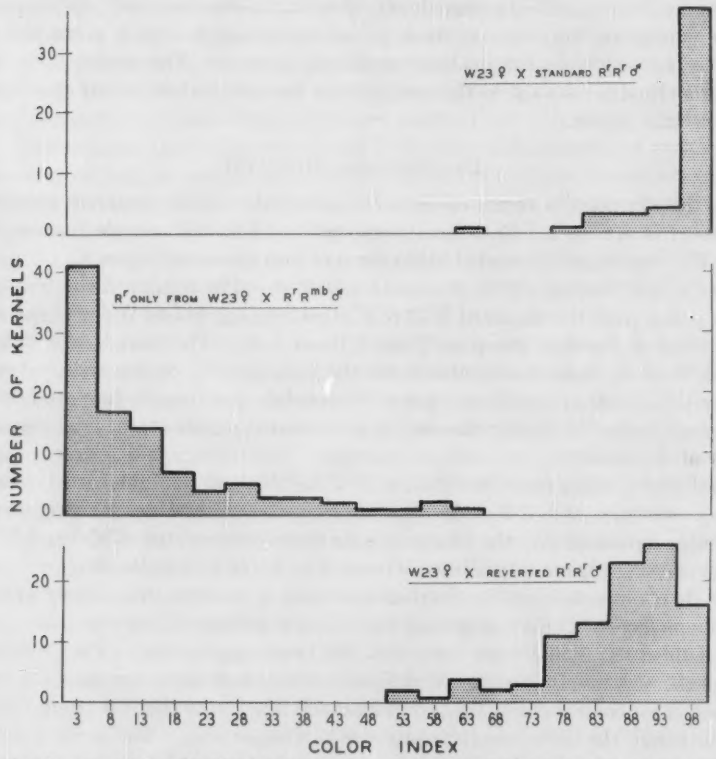
W23 ♀ X STANDARD R^rR^r♂
R^r ONLY FROM W23 ♀ X R^rR^{mb}♂
W23 ♀ X REVERTED R^rR^r♂
NUMBER OF KERNELS
COLOR INDEX
0
10
20
30
40
3 8 13 18 23 28 33 38 43 48 53 58 63 68 73 78 83 88 93 98

FIG. 1.—Representative distributions of kernels, according to aleurone color index, from testcrosses on r^gr^g plants of R^r pollen from standard R^rR^r plants (*upper right*); $R^{r:mb}$ pollen from R^rR^{mb} heterozygotes (*middle left*); and reverted $R^{r:mb}$ pollen from $R^{r:mb}R^{r:mb}$ homozygotes (*lower right*).

incomplete reversion becomes even more convincing, however, when the bias introduced into this particular comparison by the scoring method is considered. As mentioned earlier, a square in the reticule scale was counted as positive if it included *any* pigmented aleurone cells. A square containing only two or three colored cells was given the same score as a heavily pigmented one. During the examinations it became evident that a higher proportion of the squares scored as positive were solidly colored in the standard R^r testcross kernels than in those

TABLE 1*

DISTRIBUTION OF KERNELS ACCORDING TO ALEURONE COLOR INDEX FOLLOWING TESTCROSSES OF STANDARD R^rR^r, AND OF R^rR^r AND R^rR^{mb} SIBS FROM R^rR^{mb} SELFED, ON W23 r^gr^g

Testcross	Line	3	8	13	18	23	Aleurone Color Index Class Values 28	33	38	43	48	53	58	63	68	73	78	83	88	93	98	Mode
Group 1: W23 ♀ × Standard R^rR^r ♂																						
W23 × 45-89-1	1												3				2	3	3	4	35	98
-2	2												1		1	2		6	9	11	20	98
-3	3													1			1	3	3	4	37	98
-4	4																	1	4	8	37	98
Group 2: R^r Only, from W23 ♀ × R^rR^{mb} ♂																						
W23 × 45-54-1	5	49	20	14	3	2	1	2						1					1			3
-3	6	59	12	16	2	1	3	3	2	1				1								3
-10	7	45	12	9	4	4	3	2	3		1					1						3
-12	8	24	29	10	11	5	4	3		1	2	1	1		1							8
-15	9	41	17	14	7	4	5	3	3	2	1	1	2	1								3
Group 3: W23 ♀ × Reverted R^rR^r ♂																						
W23 × 45-53-5	10											1	1	5	3	10	9	13	18	25	19	93
-6	11											2		4	2	4	8	16	20	28	16	93
-14	12											2	1	4	2	3	11	13	23	26	16	93
-17	13									1		7	3	6	6	6	13	14	13	24	5	93
-25	14						2	1				2	4	6	4	9	13	9	13	19	18	93

* The ♂ parents in groups 2 and 3 are sibs. All the marbled testcross kernels both from the R^rR^{mb} plants represented in group 2 and the $R^{mb}R^{mb}$ segregates from R^rR^{mb} selfed, gave zero values for color index.

involving $R^{r:mb}R^{r:mb}$ parentage. The scoring method, therefore, tends to give an overestimate of total pigmentation in group 3, relative to group 1.

The incompleteness of the reversion in the case of the $R^{r:mb}R^{r:mb}$ plants was evident also when the unshelled ears in group 3 were laid beside those of group 1, so that an over-all visual comparison could be made. All the ears in the latter category were observed to be more darkly pigmented than any in group 3.

The bias in the scoring method which complicates an estimate of the completeness with which $R^{r:mb}$ from $R^{r:mb}$-$R^{r:mb}$ plants approximates standard R^r in pigment-producing potential applies in a lesser degree when groups 3 and 2 are compared, because of the more nearly equal distribution of reticule squares with given degrees of pigmentation. The data in Table 1 are believed to reflect rather accurately the amount of change in R^r expression which occurs in extracted $R^{r:mb}R^{r:mb}$ homozygotes relative to the pigmentation level given by the $R^{r:mb}$ male gametes arising directly from R^rR^{mb} heterozygotes.

The Uniformity of Marbled in Testcrosses.—The R^{mb} segregates on the five r^gr^g ♀ × R^rR^{mb} ♂ testcross ears represented in group 2 in Table 1, which were identified by the absence of anthocyanin in roots and first internode, gave zero indexes for color through-

out. Five sibs homozygous for marbled, when similarly tested, yielded zero values. There is no evidence from test matings on the W23 strain, therefore, that the color-determining action of R^{mb} is altered in R^rR^{mb} heterozygotes.

Phenotypic Effects of Standard R^r and $R^{r:mb}$ in Testcrosses on Marbled.—This comparison was made within the appropriate W22 inbred stocks. Kernels of the one class were obtained by putting pollen from standard R^rR^r plants on homozygous marbled individuals, and those of the second category by selfing R^rR^{mb} individuals. The latter kernels ($R^{r:mb}R^{mb}R^{mb}$) comprised the approximately 25 per cent of the total seeds formed on selfed R^rR^{mb} ears which had lightly pigmented aleurone but gave seedlings with, rather than without, anthocyanin in the roots and first internode, as would be the case with the homozygous marbled segregates. The $R^rR^{mb}R^{mb}$ kernels proved to be much more darkly pigmented than their $R^{r:mb}R^{mb}R^{mb}$ counterparts. This result shows that the change from R^r to $R^{r:mb}$ does not occur immediately upon the formation of $R^rR^{mb}R^{mb}$ aleurone cells, even though a modified phenotype of this kind ($R^{r:mb}R^{mb}R^{mb}$) is possible.

Persistence of the $R^{r:mb}$ Condition in Heterozygotes with r^g.—Plants were grown out from $R^{r:mb}{}_{r^g}r^g$ seeds from the above-mentioned W23 $r^gr^g(cc)$ ♀ × 22 R^rR^{mb} (CC) testcross ears. Five $R^{r:mb}{}_{r^g}$ (Cc) individuals were selfed, and pollen from them was applied to W23 ear shoots also. Parallel matings were made with $R^r{}_{r^g}$ (Cc) controls grown from W23 × standard 22 R^rR^r F_1 seeds. The 25 per cent of colored kernels resulting from the control testcrosses were typically dark-mottled. Four ears from the r^gr^g (cc) × $R^{r:mb}{}_{r^g}$ (Cc) matings, in contrast, showed the level of pigmentation characteristic of the parent $R^{r:mb}{}_{r^g}r^g$ (Ccc) seeds. The $R^{r:mb}$ condition arising in R^rR^{mb} plants, therefore, was maintained in these $R^{r:mb}{}_{r^g}$ heterozygotes. The fifth ear in this set of testcrosses showed wide variation in amount of aleurone mottling. The basis of this exception is not known. The selfed ears on the $R^{r:mb}{}_{r^g}$ plants carried self-colored, light-mottled, and colorless seed in the ratio 6:3:7. Since the W23 strain is homozygous recessive for two complementary genes conditioning aleurone color, c and r, this is the ratio expected if the $R^{r:mb}R^{r:mb}R^{r:mb}$ and $R^{r:mb}R^{r:mb}{}_{r^g}$ aleurone genotypes result in self-color.

Comparison of the Modified Forms of R^r Arising in Marbled and Stippled Heterozygotes.—The genetic changes which R^r undergoes in heterozygotes with marbled and stippled, although similar in that both involve a large reduction in aleurone pigment-producing action, are not alike. Both the $R^{r:st}r^gr^g$ and $R^{r:mb}{}_{r^g}r^g$ phenotypes have been produced on W23 × W22 background, but only the latter phenotype has been quantitatively characterized in this combination. Comparison of the respective R^{mb} and R^{st} effects, therefore, can be made in only general terms at present. When pollen from R^rR^{st} plants is placed in W23 ear shoots, the resulting $R^{r:st}r^gr^g$ kernels are definitely less pigmented, on the average, than are $R^{r:mb}{}_{r^g}r^g$ kernels from parallel R^rR^{mb} matings. A difference in pattern of pigmentation is discernible also, there being a greater number of relatively large colored areas in the case of $R^{r:mb}{}_{r^g}r^g$. A conspicuous difference is shown in the amount of reversion toward the standard R^r phenotype which $R^{r:st}$ and $R^{r:mb}$ undergo following extraction in homozygous form from R^rR^{st} and R^rR^{mb} plants. The kernels resulting from W23 ♀ × $R^{r:mb}R^{r:mb}$ ♂ testcrosses regularly are more darkly pigmented than those obtained from W23 ♀ × $R^{r:st}R^{r:st}$ ♂ matings. Thus, in terms of gross pigmentation, the effect on R^r of R^{mb} in heterozygotes is less than that of R^{st}; and the modi-

fied form of R^r arising in R^rR^{mb} plants, when made homozygous, shows a greater amount of reversion toward the level of action of standard R^r.

DISCUSSION

Three facts established in this study are of particular interest. First, a heritable change is found to occur regularly in R^rR^{mb} plants which is manifested in appropriate testcrosses as a markedly altered expression of the R^r phenotype. No effect on R^{mb} in the heterozygote was observed, but it is possible that this result is due to unsuitability of the particular breeding tests made for disclosure of a change in R^{mb} action. Second, the altered form of R^r, termed $R^{r:mb}$, regularly reverts partially toward the standard level of R^r pigment-producing action in homozygous $R^{r:mb}R^{r:mb}$ plants. Such reversion does not occur regularly, at least, in $R^{r:mb}r^g$ plants. The close parallel in these two respects with the effect of the stippled allele on R^r previously reported by Brink[1] is obvious. The third significant fact is that the R^r change associated with marbled, although similar to that with stippled, is not the same in terms of either the alteration occurring initially in R^rR^{mb} and R^rR^{st} heterozygotes or the amount of reversion toward standard R^r which takes place in $R^{r:st}R^{r:st}$ and $R^{r:mb}R^{r:mb}$ homozygotes. An important corollary of this difference between R^{mb} and R^{st} is that R^r in the homologous chromosome, or some factor associated with it in this transallelic phenomenon, is capable of undergoing more than one kind of change of this class.

A question of fact which the present findings raise but do not answer concerns homogeneity of the change of R^r to $R^{r:mb}$ occurring in R^rR^{mb} plants. Obvious exceptions to the rule that in such heterozygotes R^r always changes to $R^{r:mb}$ have not been encountered, and, if they occur, it is with low frequency. The induced genetic change is invariable in this sense.

Whether the new form to which R^r changes in the heterozygote is always the same is an issue which the present data bring to the fore. The group 2 families in Table 1, involving the $R^{r:mb}r^gr^g$ kernels from the W23 $\times$ R^rR^{mb} matings, show considerable dispersion for aleurone color index. This diversity, however, is not necessarily the result of genetic heterogeneity. Part of it, at least, undoubtedly arises from the sampling procedure employed in scoring the kernels. Pigmentation of the latter is highly irregular. Only a small area of constant size in the same position on each seed was measured. It became evident while the measurements were being made that the predetermined patch occasionally is unrepresentative of the kernel surface in general. No other sampling method was found, however, which was more satisfactory in all essential respects.

Granting the possibility that a significant part of the variability in the group 2 data is due to accidents of sampling the kernel surface, this explanation does not account for the relatively large dispersion for color index found in the group 3 families, based upon testcrosses of $R^{r:mb}R^{r:mb}$ segregates on the W23 strain. The conditions of scoring were alike in both groups. Yet the pigment indexes applying to the reverted $R^{r:mb}$ gametes clearly are the more diverse. The difference might be a reflection of differential reversion of $R^{r:mb}$ toward R^r. Another possibility is that the form of $R^{r:mb}$ transmitted through the eggs differs from that present in sperm, which alone was sampled in group 2. These are problems which further experiments may be expected to clarify.

The present data provide confirmatory evidence for the invariable occurrence in a localized region of a chromosome marked by R^r of specific genetic changes in response to the presence in the homologous chromosome of one or another particular allele, R^{st} or R^{mb}. The relation of this case to any of the several instances of transallelic changes encountered in other organisms is conjectural at present (cf. Brink 1956). The singular aspects of the maize phenomenon are that modified R ($R^{r:st}$ or $R^{r:mb}$) (1) differs in phenotypic effect from both the parental alleles in the heterozygotes in which it arises and (2) reverts partially toward standard R^r in homozygotes. Furthermore, the phenomenon is unique in that both the initial change of standard R^r to modified R and reversion of modified R toward standard R^r occur with extraordinary regularity under the appropriate conditions.

SUMMARY

A genetic change associated with the R^r phenotype (self-colored aleurone) invariably occurs in R^rR^{mb} maize plants (heterozygous marbled aleurone). There is no evidence that marbled is concurrently affected. The changed form of R^r, termed $R^{r:mb}$, arising in R^rR^{mb} heterozygotes is transmitted as such by $R^{r:mb}r^g$ individuals, but $R^{r:mb}$ reverts partially toward the standard level of R^r pigmentation in $R^{r:mb}R^{r:mb}$ homozygotes. The case is parallel to that previously described in R^rR^{st} (heterozygous stippled) individuals. The two altered forms of R^r differ, however, both in the aleurone pattern which they condition and the extent to which they revert toward the standard level of R^r pigmentation when made homozygous.

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*SIBLING RECOMBINANTS IN ZYGOTE PEDIGREES OF ESCHERICHIA COLI**

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Until recently, the sexual process in *Escherichia coli* was inferred only from the genetic analysis of recombination, without morphological corroboration. Cavalli's discovery[1] of a highly fertile mutant (*Hfr*) has made it possible to relate genetic exchange to a pairwise conjugation of the mating bacteria. Lederberg[2] observed and isolated conjugal pairs under phase-contrast microscopy and showed a high incidence of recombinant genotypes among their progeny. Anderson[3] has made striking electron micrographs of conjugal pairs, in which one parent was labeled by the pre-adsorption of bacteriophage. The significance of conjugal pairing has

been underlined by recent experiments on the timed interruption of the sexual process by mechanical shearing or by chemical inhibition.[3]

The present report amplifies a preliminary account[2] of the segregation of various markers in single-cell pedigrees of isolated zygotes. Particular interest attaches to the occurrence of complementary recombinants, that is, whether crossing over is reciprocal, as in *Neurospora* and *Drosophila*, or not, as has been suggested for bacteriophage.

Cultures and Methods.—The parental strains were chosen as a highly fertile combination, differing in a number of easily scored markers which had been introduced by a sequence of mutations. W-3011, an *Hfr* mating type derived from strain K-12, is motile and relatively slender; W-2401, an F^- mating type derived from another line (wg28A) of *E. coli*, is nonmotile and plumper. The conjugal pairs are thus quite characteristic under the microscope. The isolation of pairs and of cell lineages followed de Fonbrune's techniques of micromanipulation.[2, 4] After isolation, the mating pair was left undisturbed for 1 or 2 hours, by when it usually separated spontaneously. The exconjugants were then isolated to separate microdroplets and allowed to form large clones either directly or after re-isolation of the daughters for one to six fissions to establish cell lineages. These clones were then studied for their genotypic content by platings on various indicator media.

Markers and Notation.—Nine markers were scored in this cross (Table 1).

TABLE 1

CHARACTERIZATION OF 75 EXCONJUGANT CLONES

A. Sequence of loci according to incidence of clones containing the 1 allele (from *Hfr* parent).** The 1 allele is indicated below, the 0 allele above.

−	s	−	r	−	−	−	+	−	000	0000	00	F^-
Lac	V_1	*Ara*	*S*	*Mal*	*Xyl*	*Mtl*	*Gal*	*Fla*				
+	r	+	s	+	+	+	−	+	111	1111	11	*Hfr*
70	29	25	5	6	5	2	1	2				

B. ××× 0000 00 clones* (total 65)

I. Clones with One Recombinant		II. Total Incidence of This Type†	III. Clones with More than One Recombinant‡	
100*	37	45	111 & 011	7
111	6	18	111 & 100	1
110	5	8	110 & 010	1
001	2	4	100 & 010	1
011	1	8	001; 110; 100; 010	1
101	1	1	001; 100; 010	1
010	0§	5	110; 100; 010	1
000	♀	♀		

C. ××× ×××× 00 clones‖ (represented individually)

1. 111 0010
2. 111 1000; 111 1111
3. 111 1000; 111 0000
4. 100 0100; 000 0100
5. 100 0110; 100 0000; 000 0110; 000 0000‡
6. 111 1111; 111 0000; 000 1111; 000 0000‡
7. 100 0000; 100 1100; 000 1100; 000 0000‡
8. 100 1100; 100 0010; 000 1100; 000 0010

D. 000 0000 ×× clones

1. 000 0000 10
2. 000 0000 01

* The terminal group 0000 00 is to be understood. Thus 100 should be read 100 0000 00.
† This item includes the occurrences in parts B and C.
§ This class may not always have been ascertained.
‖ The terminal group 00 is to be understood. Thus 111 1000 should be read 111 1000 00.
‡ The 000 0000 00 class (♀ parent) was present in every exconjugant clone. It is written explicitly to complete possible tetrads.
** The sequence *S Mal Xyl* is preferred as minimizing apparent multiple crossing-over.

Lac, *Ara*, *Mal*, *Xyl*, *Mtl*, and *Gal* refer to the fermentation of lactose, L-arabinose, maltose, D-xylose, mannitol, and galactose, respectively; V_1 and *S* refer to the reactions (sensitive versus resistant) to phage T1 and to streptomycin. *Fla* refers to flagellation, scored by motility. However, for the purpose of this analysis, the phenotypic status of each recombinant is less important than the parental origin of the marker alleles. For simpler exposition, the allele from the F^- parent is designated 0, and its alternative from the *Hfr* parent, 1. For a marker not segregating or for another reason not specified in a given genotype, the designation will be x. Deleted segments are indicated as □□. The loci are written in the order specified in Table 1. This convention having been established, the genotypes are written as binary numbers; for example, 000 1111 00 represents Lac^- V_1^s Ara^- S^s Mal^+ Xyl^+ Mtl^+ Gal^+ Fla^-, the first three and the last two of the markers having stemmed from the F^- parent, the middle group of four (*S*, *Mal*, *Xyl*, *Mtl*) from the *Hfr*. Specific segments may be marked off by underscoring; thus 000 0000 00 refers to the *Ara-S* segment.

Hfr versus F^- represents another marker difference but was not systematically scored. However, some dozens of recombinants were all F^- with the single exception of D1 (000 0000 10) which was *Hfr*, in accord with Cavalli and Jinks's demonstration of linkage of *Hfr* to *Gal*.[5]

Both parents were sensitive to the temperate phage lambda, except that the Mal^- marker was associated with poor adsorption of the phage.[6]

Experimental Results and Discussion.—Table 1 exhibits the recombinant progeny from 75 conjugal pairs. About 125 other pairs that were isolated and gave a viable F^- exconjugant produced no detectable recombinants. The *Hfr* exconjugant was usually viable as well; however, all the recombinants appeared in the progeny of the F^- exconjugant. This asymmetry implies the unilateral transfer of genetic material from the *Hfr* to the F^- cell and justifies the description of the two mating types as ♂ and ♀, respectively.[7] However, *Hfr* × *Hfr* crosses are also fertile, though F^- × F^- are not. The *Hfr* is therefore ambivalent, but in crosses with F^- (obligate ♀) it can function only as a ♂. By analogy with the mating systems of other fungi, obligate ♂♂ may be supposed to exist but have not yet been noticed in *E. coli*.

In addition to recombinants, each F^- exconjugant contained a proportion, usually more than half, of the F^- parental genotype, viz., 000 0000 00. In pedigreed clones, at the second fission, usually three of the cells were pure 000, etc., while the fourth was still generating both parental and recombinant genotypes, these becoming pure in cells at the third or fourth fission. Figure 1 shows an instance of prolonged delay in segregation, with recombinants in three of the subclones at the four-cell stage. These results are readily understood by the regular assortment of three unfertilized from one fertilized nucleus and its meiotic products in the lineage of the multinucleate F^- exconjugant, complicated by occasional slippage and irregular assortment of nuclei. No persistent heterokaryons were observed in this study, nor could they be expected in a short bacillary form, whereas they are expected and are found in the filamentous streptomycetes.[8]

The various markers in Table 1 are arranged according to their segregation ratio, i.e., the relative incidence of the 1 allele among the exconjugant clones. As shown, this incidence varied from 1 each for *Gal* and *Fla* to 70 for *Lac*. This disparity con-

tradicts the assumption of random segregation of unselected markers which was postulated in the initial analysis of segregation and linkage in *E. coli*.[9] The disparity does accord with the bias found in unselected progenies from persistent heterozygotes[10, 11] and is now explained by the loss of segments from the ♂ chromosome, either before fertilization,[3] or after,[11] or both.

The 1 allele of each of the 9 markers of these parents has been recovered at least once, i.e., the ♂ parent *can* transmit any element. The ♂ exconjugants of exceptional zygotes such as D1, D2, and others were examined for a persistent alteration in the segregation pattern of further crosses, but none was found. Within this series, there was no instance (barring one creditable to a possible contamination) of the reappearance of the whole 111 1111 11 genotype in the progeny of a ♀ exconjugant. Whether or not the entire 111, etc., genotype is transmitted to the zygote, it therefore experiences either obligatory recombination or elimination of a chromosome segment at some stage, be this before or after fertilization.

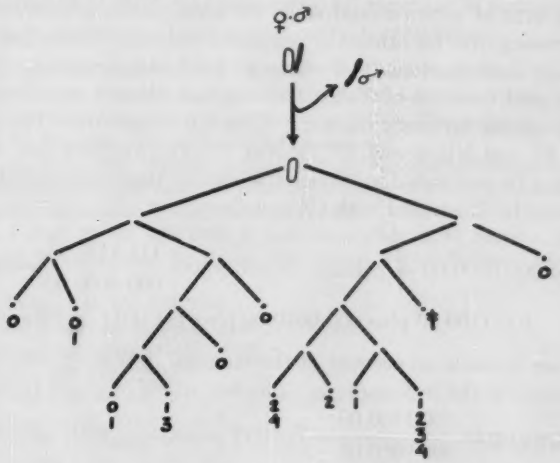

FIG. 1.—Cell lineage from a conjugal pair (C8). The lineage is abbreviated: where a subclone was pure, only the pure parent cell is shown. Genotypes (see Table 1): ♂ = 111 1111 11. ♀ = 0 = 000 0000 00. *1* = 000 1100 00; *2* = 100 0010 00; *3* = 100 1100 00; *4* = 000 0010 00. † = died.

Most of the clones contained only one detectable recombinant. However, as some pedigreed cells were inviable from technical mishaps or from no overt injury, this class may be overestimated. The incidence of these single-recombinant clones tells against the frequent occurrence of double fertilizations. The mating complexes chosen for isolation are, however, selected as simple pairs, and multiple fertilizations could still occur in the more complicated clumps which are also seen.

The distribution of types in part BIII suggests considerable coincidence of interchanges. Thus we find six clones with 111 alone, just one with 011, but seven with both together. Part C also points to an interaction between recovery of 111 and of further markers. That is, crossing over in the 000 0000 00 region is correlated with crossovers in other regions. This type of coincidence has been stressed in earlier reports[5] and can again be interpreted as a result of sporadic pairing, either

from poor synapsis in a complete heterozygote, or from the prezygotic loss of some chromosome segments.

Twenty clones had from two to four recombinants, none more than four, which suggests a regular mechanism of meiosis with a four-strand stage. Clones C5 through C8 are especially striking, as they comprise four different tetratype tetrads with partly complementary elements. For example, C6 includes the complementary set: 111 1111; 000 0000; 111 0000; and 000 1111. Another example of complementarity, viz., the reciprocal crossovers S^s Mal^+ Xyl^- and S^r Mal^- Xyl^+, is seen in C8. However, the suggestion that crossing over is inherently reciprocal must be qualified in view of the small numbers and the possible role of coincidence.

The frequency of clones with three or four recombinants poses an additional problem. Of the four tetratypes, three show the same crossover, $\underline{100}$, etc., in two combinations. For example, C8 has $\underline{100}$ 1100 and $\underline{100}$ 0010. Three possibilities are forwarded: a four-stranded heterozygote, with intense coincidence of crossing over; crossing over at a two-strand stage for some markers, followed by typical four-strand crossing over for others; or reiterated meiosis. As the last formulation also accounts for another anomaly, it will be set forth in more detail.

The abbreviated notation of Table 1 should not obscure the fact that no one clone was segregating for every marker. Even C6 is pure Gal^+ Fla^-, while C5 is pure for *Ara*, V_1, and *Mtl* as well; C7 for *Ara*, V_1, *Xyl*, and *Mtl*; and so on. Some mechanism must be postulated to furnish 0 alleles for these recombinant genotypes. One scheme may be illustrated with C8 as follows:

$$111\ 1111\ 11 \times 000\ 0000\ 00 \rightarrow \text{primary heterozygote } \frac{111\ 1111\ 11}{000\ 0000\ 00} \rightarrow \text{first meiosis} \rightarrow$$

$$100\ 1100\ 00 \text{ plus } 000\ 0010\ 00 \text{ plus } 111\ 0011\ \square\square \text{ plus } 011\ 1111\ \square\square.$$

The last two are inviable on account of the terminal deficiency, which stems from breakage proximal to the two markers. The two viable products rejoin to give the secondary heterozygote $\frac{100\ 1100\ 00}{000\ 0010\ 00}$. Further reduction, with crossing over at a four-strand stage in the region $\underline{100}$ $\overline{1100}$ 00, gives the four products: 100 1100 00; 000 0010 00; 100 0010 00; and 000 $\overline{1100}$ 00.

The secondary heterozygote can be formulated by an alternative scheme, on the supposition of prezygotic loss of segments to give a fractional heterozygote (merozygote[3]):

$$\underline{111}\ 1111\ \underline{11} \times 000\ 0000\ 00 \rightarrow \frac{1\square\square\,111\square\ \square\square}{000\ 0000\ 00}.$$

The merozygote would then be completed by partial replication from the 000, etc., template to give

$$\frac{100\ 1110\ 00}{000\ 0000\ 00}$$

followed, or accompanied, by crossing over to give $\frac{100\ 1100\ 00}{000\ 0010\ 00}$,

which is then reduced by a regular four-strand meiosis. However, as partial replication can be posited as the mechanism of crossing over, the two versions are formally similar and cannot be distinguished in the present experiments. On both versions, crossing over takes place between *Mal* and *Xyl* during the primary cycle.

The formation of secondary heterozygotes has been postulated previously[10] to account for demonstrable homozygosity of *Lac* in some persistent diploids and for "twin prototroph" recombinants. Sporadic automixis is characteristic of persistent diploids[10] and heterogenotes.[12] Iterated meiosis is therefore a recurrent feature of recombination in *E. coli*.[13]

Summary.—Individual mating pairs were isolated from highly fertile matings of *E. coli*, *Hfr* × F^-. All the recombinants were found in the progeny of the F^- exconjugant, which is therefore designated as ♀. The segregation of markers in the cell lineage indicated (1) the loss of certain chromosome segments, before or after fertilization or both; (2) coincidence of crossing over, suggestive of sporadic pairing; (3) possible correlation of reciprocal crossovers; (4) recycling of meiosis; and (5) an interval of nuclear assortment from a temporary heterokaryon.

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*CONGRUENCES INVOLVING COMBINATIONS OF THE BERNOULLI AND FIBONACCI NUMBERS**

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In this note we shall give some congruences involving sums of products of Bernoulli and Fibonacci numbers and sums of products of Bernoulli and Lucas numbers. The apparently new congruences,

$$\sum_{k=0}^{\overline{p-3}} B_{2k}u_{2k} \equiv {}^1/_2 \pmod{p} \qquad \text{if} \qquad p = 5l \pm 1,$$

$$\sum_{k=0}^{\overline{p-3}} B_{2k}u_{2(k+1)} \equiv 1 \pmod{p} \qquad \text{if} \qquad p = 5l \pm 2, \tag{1}$$

are special cases of the general congruences obtained below. The general congruences are obtained from identities involving these numbers, but in this note we shall state these identities without proof. Also, we shall only outline the method used to obtain the congruences.

If a and b are integers and $a > b$, we shall find it convenient to define

$$\sum_{k=a}^{k=b} f(k) = 0.$$

Also, the greatest integer not exceeding $n/2$ will be denoted by $\bar{n}$. In the following discussion p always denotes an odd prime, and all congruences are modulo p. We shall use the Blissard symbolic notation: if there exists a positive number T such that

$$f(t) = \sum_{n=0}^{\infty} a_n t^n/n! \qquad \text{for} \qquad |t| < T,$$

then we shall write

$$f(t) \overset{*}{=} e^{at}.$$

The Bernoulli polynomial, $B_n(x)$, and the Euler polynomial, $E_n(x)$, are defined symbolically by the relations

$$\frac{t}{2} e^{(x - {}^1/_2)t} \operatorname{csch} \frac{t}{2} \overset{*}{=} e^{B(x)t}$$

and

$$e^{(x - {}^1/_2)t} \operatorname{sech} \frac{t}{2} \overset{*}{=} e^{E(x)t},$$

respectively. We define two other sequences of polynomials symbolically:

$$2e^{xt/2} \sinh \frac{t}{2} \stackrel{*}{=} e^{F(x)t} \tag{2}$$

and

$$2e^{xt/2} \cosh \frac{t}{2} \stackrel{*}{=} e^{L(x)t}. \tag{3}$$

Hence, if x is a number and n is a positive integer,

$$F_n(x) = \frac{(x+1)^n - (x-1)^n}{2^n},$$

and

$$L_n(x) = \frac{(x+1)^n + (x-1)^n}{2^n}.$$

If $n = 0$, then $F_0(x) = 0$, and $L_0(x) = 2$ for every number x.

The sequence of Fibonacci is defined by the difference equation,

$$f_{n+1} = f_n + f_{n-1}$$

with $f_0 = 0$ and $f_1 = 1$. The sequence of Lucas[1] satisfies the same difference equation, but in this case $f_0 = 2$ and $f_1 = 1$. We shall denote the nth Fibonacci number by u_n and the nth Lucas number by v_n. It has been shown[2] that if r is a positive integer and $\alpha_r = v_r/\sqrt{5}u_r$, then

$$u_r{}^n(\sqrt{5})^{n-1} F_n(\alpha_r) = u_{rn}, \tag{4}$$

and

$$u_r{}^n(\sqrt{5})^n L_n(\alpha_r) = v_{rn}. \tag{5}$$

We shall define the Bernoulli number, B_n, to be $B_n(0)$. It has been shown[3] that if m is a positive integer and n is a nonnegative integer, then

$$\sum_{k=0}^{\bar{n}} \binom{n}{2k} B_{2k} m^{-2k} F_{n-2k}(x) - \frac{n}{2m} L_{n-1}(x) = \frac{n}{m} \sum_{s=1}^{m-1} \left(\frac{x}{2} + \frac{s}{m} - \frac{1}{2}\right)^{n-1}. \tag{6}$$

We note that if $x = 1$, equation (6) reduces to the well-known Bernoulli summation formula. If we define the Euler number, E_n, to be $2^n E_n\left(\frac{1}{2}\right)$, then, analogous to equation (6), we may obtain, for m odd,

$$\sum_{k=0}^{\bar{n}} \binom{n}{2k} (2m)^{-2k} E_{2k} L_{n-2k}(x) = 2 \sum_{s=0}^{m-1} (-1)^s \left(\frac{x}{2} + \frac{2s+1}{2m} - \frac{1}{2}\right)^n. \tag{7}$$

If m is an even positive integer, then

$$\sum_{k=0}^{\overline{n-1}} \binom{n}{2k+1} (2k+1)(2m)^{-2k} E_{2k} F_{n-2k-1}(x) = 2n \sum_{s=0}^{m-1} (-1)^{s+1} \left(\frac{x}{2} + \frac{2s+1}{2m} - \frac{1}{2}\right)^n. \tag{8}$$

If m and n are positive integers and $x \neq 0$, we define

$$f_1(m, n, x) = \sum_{t=0}^{\overline{m-3}} F_{n-1}\left(\frac{2t+1}{mx}\right)$$

and

$$f_2(m, n, x) = \sum_{t=0}^{\overline{m-2}} F_{n-1}\left(\frac{2t}{mx}\right).$$

It is easily shown that

$$\sum_{s=1}^{m-1}\left(\frac{x}{2} + \frac{s}{m} - \frac{1}{2}\right)^{n-1} = \begin{cases} x^{n-1} f_1(m, n, x), & m \text{ odd}, \\ x^{n-1} f_2(m, n, x) + \left(\frac{x}{2}\right)^{n-1}, & m \text{ even}. \end{cases} \tag{9}$$

Finally, we note that if a and b are positive integers and $(a, b) = 1$, p is an odd prime, and $p + (b^2 - 5a^2)$, then

$$F_{p-2}\left(\frac{a\sqrt{5}}{b}\right) \equiv \begin{cases} 4b^2(b^2 - 5a^2)^{-1} & \text{if} \quad p = 5l \pm 1, \\ 4b^2(b^2 + 15a^2)(b^2 - 5a^2)^{-2} & \text{if} \quad p = 5l \pm 2. \end{cases} \pmod{p} \tag{10}$$

If $p \mid (b^2 - 5a^2)$, then

$$F_{p-2}\left(\frac{a\sqrt{5}}{b}\right) \equiv 1. \pmod{p} \tag{11}$$

In order to obtain the congruences which we are seeking, set $n = p - 1$ and $x = 1/\sqrt{5}$ in equation (6). It is easily shown[4] that

$$u_{p-1-2k} \equiv \begin{cases} -u_{2k} & \text{if} \quad p = 5l \pm 1, \\ u_{2(k+1)} & \text{if} \quad p = 5l \pm 2. \end{cases} \pmod{p}$$

If $(m, p) = 1$, and $p = 5l \pm 1$, then, after some simplification, we have

$$\sum_{k=0}^{\overline{p-3}} B_{2k} m^{-2k} 5^k u_{2k} + \frac{1}{2m} \equiv \begin{cases} \frac{1}{m} f_1(m, p-1, 1/\sqrt{5}), & m \text{ odd}, \\ \frac{1}{m} f_2(m, p-1, 1/\sqrt{5}) + \frac{2}{m}, & m \text{ even}. \end{cases} \pmod{p} \tag{12}$$

If $(m, p) = 1$ and $p = 5l \pm 2$, then

$$\sum_{k=0}^{\overline{p-3}} B_{2k} m^{-2k} 5^k u_{2(k+1)} + \frac{2}{m} \equiv \begin{cases} -\frac{1}{m} f_1(m, p-1, 1/\sqrt{5}), & m \text{ odd}, \\ -\frac{1}{m} f_2(m, p-1, 1/\sqrt{5}) - \frac{2}{m}, & m \text{ even}. \end{cases} \pmod{p} \tag{13}$$

With the results of congruences (10) and (11), we may simplify the right-hand members of (12) and (13) for given p and m.

If we set $n = p - 1$, $x = \sqrt{5}$, and $(m, p) = 1$ in equation (6), then results[5] similar to those in equations (4) and (5), together with equations (9), yield, for $p = 5l \pm 1$,

$$\sum_{k=0}^{\overline{p-3}} B_{2k} m^{-2k} u_{2k} - \frac{1}{2m} \equiv \begin{cases} \frac{1}{5m} f_1(m, p-1, \sqrt{5}), & m \text{ odd}, \\ \frac{1}{5m} f_2(m, p-1, \sqrt{5}) + \frac{2}{5m}, & m \text{ even}. \end{cases} \tag{14}$$

If $p = 5l \pm 2$, we have

$$\sum_{k=0}^{\overline{p-3}} B_{2k} m^{-2k} u_{2(k+1)} - \frac{1}{m} \equiv \begin{cases} \frac{1}{5m} f_1(m, p-1, \sqrt{5}), & m \text{ odd}, \\ \frac{1}{5m} f_2(m, p-1, \sqrt{5}) + \frac{2}{5m}\, 2\,, & m \text{ even}. \end{cases} \tag{15}$$

For example, if $m = 1$, we obtain the simple congruences given in (1).

If we take $n = p - 2$ and $x = \sqrt{5}$ in equation (6), then we may obtain congruences involving Bernoulli numbers and Lucas numbers. If $p = 5l \pm 1$, then

$$\sum_{k=0}^{\overline{p-3}} (2k+1) B_{2k} m^{-2k} v_{(2k+1)} - \frac{3}{m} \equiv \begin{cases} \frac{2}{5m} f_1(m, p-2, \sqrt{5}), & m \text{ odd}, \\ \frac{2}{5m} f_2(m, p-2, \sqrt{5}) + \frac{8}{5m}, & m \text{ even}. \end{cases} \tag{16}$$

If $p = 5l \pm 2$, in congruence (16) replace v_{2k+1} by v_{2k+3} and replace the term $3/m$ by $7/m$.

Congruences in which the Euler and Lucas numbers or Euler and Fibonacci numbers appear may be obtained from equations (7) and (8) by similar methods.

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[1] Different sequences of numbers have been called Lucas sequences. We are using the terminology of G. H. Hardy and E. M. Wright, *The Theory of Numbers* (London, 1954), pp. 148–150.

[2] R. P. Kelisky, *Scripta Math.*, Vol. 23, 1957.

[3] *Ibid.*

[4] Hardy and Wright, *loc. cit.*

[5] Kelisky, *loc. cit.*

GENERAL SOLUTION FOR THE STRESSES IN A CURVED MEMBRANE

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For Cauchy's equations of equilibrium for a free continuous medium,[1] viz.,

$$t^{km}{}_{.m} = 0, \qquad t^{km} = t^{mk}, \tag{1}$$

where the comma denotes the covariant derivative, the general tensorial solution for a flat space of n dimensions has been known for some time.[2] For a curved space, such as an ordinary two-dimensional surface, a solution in tensorial form is not known, though various special results are available.[3]

In a penetrating memoir, L. Finzi[4] has exploited the long recognized similarity between the forms of certain known general solutions of equations (1) and the forms of the conditions of compatibility for the same space. He has shown that if the general solution is known, proper application of the converse of the principle of virtual work[5] yields the conditions of compatibility. Second, after assuming a certain form for the general solution of equations (1), he has determined the coefficients in that form explicitly by comparison with the result just mentioned, on the assumption that the conditions of compatibility are known. He has inferred that the solution so obtained is general and has illustrated it by exhibiting, for the first time, the general tensorial solution for a surface of revolution.

It does not detract from the originality and value of L. Finzi's work to point out that, while both his results are correct, the second inference rests on the general form assumed but not demonstrated for the solution of equations (1). Moreover, this second result may be obtained directly and in simpler form from the usual principle of virtual work. Indeed, that principle asserts that the tensor t^{km} satisfies equations (1) if and only if

$$\int t^{km} d_{km} dv = 0 \tag{2}$$

for all fields d_{km} such that $d_{km} = c_{(k,\,m)}$ for some vector c_k. Here and in what follows we systematically neglect boundary integrals as of no effect on the differential equations we seek.[6] This form of the principle, since it rests only on Green's theorem, is valid in any space where covariant differentiation is defined. Assume that the conditions of compatibility for the system $d_{km} = c_{(k,\,m)}$ have the explicit form

$$0 = \alpha^{km} d_{km} + \beta^{kmr} d_{km,\,r} + \gamma^{kmrs} d_{km,\,rs} + \delta^{kmrsp} d_{km,\,rsp} + \ldots, \tag{3}$$

where the tensors $\alpha^{km}, \beta^{kmr}, \ldots$, are symmetric in the first two indices. Introducing a multiplier A, we may then replace (2) by

$$\int [t^{km} d_{km} - A(\alpha^{km} d_{km} + \beta^{kmr} d_{km,\,r} + \ldots)] dv = 0, \tag{4}$$

where the variation of d_{km} is now unrestricted. By Green's theorem follows the equivalent statement

$$\int [t^{km} - \alpha^{km}A + (\beta^{kmr}A)_{,r} - (\gamma^{kmrs}A)_{,rs} + \ldots]d_{km}dv = 0. \tag{5}$$

Hence

$$t^{km} = \alpha^{km}A - (\beta^{kmr}A)_{,r} + (\gamma^{kmrs}A)_{,rs} - (\delta^{kmrsp}A)_{,rsp} - \ldots. \tag{6}$$

We have shown, then, that *the general solution of (1) is of the form (6) whenever the conditions of compatibility have the form (3).* This is equivalent to the result inferred by L. Finzi in the special case when the sum (3) terminates after the number of terms actually written down. The stress function A appears as the multiplier for the constraint expressing the compatibility of the deformation with a virtual displacement field for the space considered. It is perhaps necessary to emphasize that the above argument includes a *proof of completeness;* moreover, unlike the usual proofs based on the theorem of representation of a solenoidal field in terms of a vector potential, this proof is not merely local but, to the extent that the method of multipliers is valid in the calculus of variations, proves the existence of the stress functions *in the large.*

The form (3) of the conditions of compatibility is appropriate to spaces of two dimensions. For spaces of three dimensions, the tensors α^{km}, β^{kmn}, ..., are to be replaced by tensors α^{ijkm}, β^{ijkmr}, ... Accordingly, the scalar multiplier A in (4), and hence the stress function A in the solution (6), is to be replaced by a tensor A^{ij}.

Since the process applies equally when the coefficients in (3) are tensors of any order, knowledge of the conditions of compatibility is shown to be equivalent to the integration of (1), for any space where covariant differentiation is defined. There may, of course, be spaces where neither problem admits a solution.

To apply formula (6) to membranes,[7] we need the explicit form of the conditions of compatibility (3) in a Riemannian space of two dimensions with covariant metric tensor a_{km}. For a *surface of constant curvature* K, B. Finzi[8] has shown that

$$\begin{aligned} \alpha^{km} &= Ka^{km}, \qquad \beta^{kmr} = 0, \\ \gamma^{kmrs} &= -e^{s(k}e^{m)r}, \end{aligned} \tag{7}$$

where $\sqrt{a}e^{ks}$ is the permutation symbol such that $\sqrt{a}e^{12} = +1$ and where all coefficients of higher order vanish. Hence the general solution (6) for membranes of constant curvature is

$$t^{km} = e^{kp}e^{mq}A_{,pq} + KAa^{km}, \tag{8}$$

which is the result given by B. Finzi.[2] For a *surface applicable upon a surface of revolution,* B. Finzi[8] has shown that

$$\begin{aligned} \alpha^{km} &= K_{,p}K^{,p}a^{km} + K^{,k}K^{,m}, \\ \beta^{kmr} &= KK^{,r}a^{km} \qquad \gamma^{kmrs} = 0, \\ \delta^{kmrsp} &= -e^{s(k}e^{m)r}K^{,p}, \end{aligned} \tag{9}$$

while all coefficients of higher order vanish. Hence the general solution (6) for such a surface is

$$\begin{aligned} t^{km} = {} & [(K_{,p}K^{,p} - KK_{,p}{}^{,p})a^{km} + K^{,k}K^{,m} + e^{s(k}e^{m)r}K_{,rsp}{}^{,p}]A \\ & + [-KK^{,r}a^{km} + e^{s(k}e^{m)q}K_{,qs}{}^{,r} + (e^{r(k}e^{m)q} + e^{q(k}e^{m)r})K_{,qp}{}^{,p}]A_{,r} \\ & + [(e^{r(k}e^{m)q} + e^{q(k}e^{m)r})K_{,q}{}^{,s} + e^{s(k}e^{m)r}]A_{,rs} \\ & + e^{s(k}e^{m)r}K^{,p}A_{,rsp}, \end{aligned}$$

equivalent to the result inferred by L. Finzi.[4]

For a general surface not falling into the above-mentioned special classes, B. Finzi[5] has obtained a condition of compatibility consisting not in a single scalar equation but in a system of differential order 4 and tensorial order 2. Using this result and the method described in the paragraph starting after equation (6), for the stresses in a membrane of arbitrary form we could easily exhibit a general solution in terms of the fourth derivatives of a second-order tensor of stress functions. However, the result of Storchi[3] in geodesic co-ordinates indicates the existence of a solution in terms of fifth derivatives of a single stress function. Conversely, from this fact and the first result of L. Finzi[4] it follows that there exists a single scalar condition of compatibility, of fifth order, for an arbitrary curved surface. If that condition can be calculated, then the result of this paper will yield at once the general tensorial solution for the stresses in a membrane.

It should be possible to approach the problem of deriving conditions of compatibility for the system $d_{km} = c_{(k,\,m)}$ by a method valid in a general affine space. Apparently it is difficult; I have not been able to find any literature concerning it.

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[1] Various mechanical interpretations are possible. In the simplest, t^{km} is the stress tensor in a medium in equilibrium subject to no body force. In other interpretations, t^{km} includes the momentum transfer stress of a steady motion and/or a stress equivalent to a conservative body force. In still others, t^{km} is a combined stress-momentum tensor of a not necessarily steady motion. For a medium subject to arbitrary load F^k, $(1)_1$ is to be replaced by $t^{km}{}_{,m} + F^k = 0$; to solve this equation when the general solution of $(1)_1$ is known, only a particular integral is required.

For the case of a two-dimensional surface imbedded in three-dimensional Euclidean space, the solution of (1) constitutes the classical membrane problem, on which there is a literature dating back to Beltrami. In this context the equilibrium of tangential forces and of moments is expressed by (1), while the equilibrium of normal forces is expressed by the linear algebraic equation

$$b_{km}t^{km} + F = 0, \tag{A}$$

where b_{km} is the second fundamental form and F is the normal pressure. When the general solution of (1) in terms of a stress function is put into eq. (A), this latter becomes a differential equation for the stress function.

Mention should be made of the solution given by Pucher (1934), the subject of a recent exposition by L. Finzi, *Rend. Ist. Lombardo sci.*, **88**, 907–916, 1955. In this solution, based on a special oblique co-ordinate system, eqs. (1) and (A) are combined at the start, so that the intrinsic problem is not treated.

Results based on (1) are not to be confused with those appropriate to a *shell*, which may sustain cross-forces, bending moments, and twisting moments, as well as surface stresses.

[2] The result is due to R. F. Gwyther, *Mem. Proc. Manchester Lit. & Phil. Soc.*, Vol. **56**, No. 10, 1912; and B. Finzi, *Rend. accad. naz. Lincei*, **19**, 578–584, 620–623, 1934. An elegant general proof was recently given by W. S. Dorn and A. Schild, *Quart. Appl. Math.*, **14**, 209–213, 1956. There is a burgeoning literature on the subject.

[3] The general solution in geodesic co-ordinates was derived by E. Storchi, *Rend. accad. naz. Lincei*, **8**, 116–120, 326–331, 1950. A tensorial solution for spaces of constant curvature had been asserted by B. Finzi, *loc. cit.*

[4] *Rend. accad. naz. Lincei*, **20**, 205–211, 338–342, 1956.

[5] In the form given, e.g., by Dorn and Schild, *loc. cit.*

[6] The reader who doubts this may dispose of surface integrals rigorously by an argument parallel to that used in the cited work of Dorn and Schild.

[7] It should be unnecessary to remark that restricting the virtual displacements in (2) to be vectors in the two-dimensional Riemannian space in no way restricts actual displacements such as the membrane may have undergone in order to assume the figure of equilibrium in which (1) is satisfied.

[8] B. Finzi, *Rend. Ist. Lombardo sci.*, **63**, 975–982, 1930.

SOME STATISTICAL OBSERVATIONS ON A CO-OPERATIVE STUDY OF HUMAN PULMONARY PATHOLOGY

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Because of the reported persistent increase in the incidence of cancer of the lung, Dr. Stanley P. Reimann, of the Lankenau Hospital in Philadelphia, arranged for the Scientific Advisory Board of the Tobacco Industry Research Committee a co-operative study by a number of pathologists in different centers across the country to examine systematically the conditions at autopsy in the bronchial mucosae of run-of-the-mill (not specially selected) cases. He has given a general account of the design of the study and of its major findings.[1] It is the object of the present paper to give some of the statistical details, with comments upon them so far as the total picture can be put together at the time of writing.

The conditions found on the pathologists' slides were classified in ascending order of pathogenicity from normal to carcinoma in six ranks: 0, normal, 1, hyperplasia; 2, squamous metaplasia; 3, atypical metaplasia; 4, carcinoma-in-situ; 5, carcinoma—based upon the worst condition found anywhere on the slide. It is obvious that in such a classification a considerable degree of judgment is involved, as the line between adjacent classes is not so sharply definable as to be interpreted in an identical manner by different judges; indeed, it is not always possible for a single person to repeat precisely a classification he has previously made.[2, 4] Thus the pooling of data is almost certain to involve what statisticians know as "mixed classification," but it is one way to get a sort of standard with which smaller samples may be compared instead of comparing each and every sample with every other.

TABLE 1

MALES

Age	Slides	Percentage Distribution Over Classes					
		0	1	2	3	4	5
Under 25	666	86.6	6.0	6.3	1.1	...	...
25–44	1,240	70.2	11.9	11.5	5.7	...	0.7
45–64	3,982	70.0	12.4	14.0	2.3	0.1	1.3
65 and up	2,702	68.8	12.8	13.4	3.2	0.2	1.6
Total	8,590	70.9	12.0	12.8	3.0	0.1	1.2
25 and up	7,924	69.6	12.5	13.4	3.2	0.1	1.3

The pooled data for males by age are given in Table 1. As Dr. Reimann pointed out[1] there is no substantial increase in the percentages in each class after age 25, except in class 5 (carcinoma), where it would be expected.

If it is desired to compare males and females, it will be necessary to restrict the coverage to those places which sent in data for both men and women, because it so happens that those pathologists reporting on only male autopsies had a slightly

different distribution of percentages by classification than those reporting both. It

TABLE 2F

FEMALES

AGE	SLIDES	PERCENTAGE DISTRIBUTION OVER CLASSES					
		0	1	2	3	4	5
Under 25	400	91.8	6.0	2.0	0.2	...	...
25–44	803	88.2	5.2	6.2	0.4	...	...
45–64	1,157	81.8	8.3	7.4	1.4	0.2	0.9
65 and up	1,301	86.2	7.3	5.2	0.9	...	0.4
Total	3,661	85.9	7.0	5.8	0.9	0.1	0.4
25 and up	3,261	85.1	7.1	6.3	1.0	0.1	0.5

TABLE 2M

CORRESPONDING MALES

AGE	SLIDES	PERCENTAGE DISTRIBUTION OVER CLASSES					
		0	1	2	3	4	5
Under 25	615	86.5	6.2	6.2	1.1	....	...
25–44	928	73.3	11.4	11.3	3.2	....	0.8
45–64	2,871	74.1	10.1	13.1	1.6	0.1	1.0
65 and up	2,044	72.6	11.6	13.0	2.2	0.1	0.6
Total	6,458	74.7	10.4	12.1	2.0	0.04	0.8
25 and up	5,843	73.4	10.9	12.8	2.1	0.1	0.8

is seen that at all ages women have higher percentages of normal slides and lower percentages in each other class except for an insignificant excess in carcinoma-in-situ at ages 45–64 based on two cases. Further, it is seen that, for women as for men, there are no really substantial changes after age 25.

As to the slides for cases with primary lung cancer in males, the numbers and percentages are given in Table 3. Comparison with the last line of Table 1 shows that

TABLE 3

AGE	SLIDES	PERCENTAGE DISTRIBUTION OVER CLASSES					
		0	1	2	3	4	5
25 and up	596	50.3	8.4	18.6	5.5	1.2	16.0

the percentages of normal and hyperplasia are down, whereas the percentages of metaplasia, squamous, and atypical, and of carcinoma-in-situ and carcinoma are up, as might be expected. Even if adjustment be made for the 16 per cent of carcinoma by dividing by 0.84, the statement remains true that normal and hyperplasia are down and metaplasia, squamous, and atypical, and carcinoma-in-situ are up; for the percentages become 59.9, 10.0, 22.1, 6.5, and 1.5 compared with 69.6, 12.5, 13.4, 3.2, 0.1. Unfortunately, these results cannot be compared with Auerbach's[1] because his classification and his method of collecting data are both different from those employed in the study.

Corresponding to the last lines of Table 1 (all males 25 and up) and Table 2F (all females 25 and up) the different cities for which we have records of individual percentages give the results in Table 4 for the means and for the standard de-

TABLE 4

	PERCENTAGE DISTRIBUTION OVER CLASSES					
	0	1	2	3	4	5
Males	65.2 ± 13.5	15.6 ± 13.1	15.0 ± 5.3	2.9 ± 2.0	.15 ± .16	1.1 ± 1.0
Females	81.2 ± 9.2	12.0 ± 10.4	5.8 ± 2.9	0.7 ± 1.0	.03 ± .02	0.2 ± 0.4

viations of the distributions about them. Although the means of the percentages do not differ materially from the mean percentages, the relatively large estimated standard errors of the distributions about them are noteworthy and signify either that the pathological conditions in the different places are different or that the different pathologists were classifying differently or both, and there is no way to distribute the variation over these two sources.

An experiment was made by asking each of the twelve participating pathologists to send in a few slides, some typical and others problematical. From these, Dr. Reimann selected 40 which were classified independently by all twelve. There were therefore 480 items spread over the 6 classes 0–5, respectively, as 48, 120, 223, 50, 7, 32. Division by 12 gives the average values 4, 10, 18.6, 4.2, 0.6, and 2.7. For simplicity, one may combine 0 and 1 (normal and hyperplasia) to get 14, keep 2 (squamous metaplasia) at 18.6, and combine the three most serious conditions 3, 4, and 5 at 7.4. A chi-square on the table of 3×12 observed values gives about 75 for 22 degrees of freedom, which is very significant of differences in the classification.[3]

There is no way to estimate what would have been the differences or their significance, had the twelve participating pathologists each read the 12,000 or so slides in the main study or any random sample of them sufficiently large to establish a reasonably stable set of data for the purpose of comparison between them. If one were satisfied to make three groups, as above, namely, classes 0 and 1, class 2, and classes 3, 4, and 5, it would be well to have 400 or 500 randomly selected slides read by several pathologists.

Another way to deal with the matter is to make a correlation table of the pairs of classifications by the 66 pairs of classifiers. The table may be constructed in a symmetrical way, resulting in $132 \times 40 = 5{,}280$ entries, each reader appearing as Reader I and as Reader II.

TABLE 5

SYMMETRIC CORRELATION TABLE FOR READERS

		1	2	3	4	5	Total
0	190	189	115	34	..	...	528
1	189	584	409	129	1	8	1,320
2	115	409	1,696	208	19	6	2,453
3	34	129	208	138	13	28	550
4	...	1	19	13	12	32	77
5	...	8	6	28	32	278	352
Total	528	1,320	2,453	550	77	352	5,280

In this table 54.9 per cent of the entries are in the main diagonal, showing that degree and only that degree of agreement between the pairs of pathologists. If the two adjacent diagonals be included, the result is 87.1 per cent of the entries, indicating that 12.9 per cent of the entries were for cases in which two pathologists differed by two or more classes in their estimates of the most serious condition on a slide.[4] One cannot convert the correlation table into a standard correlation coefficient without implying that the six classifications 0–5 are proper numerical measures of the relative pathogenicities.[5] But if one uses this assumption, $r_{I,\,II} = 0.692$ with 5 per cent and 95 per cent points at $r = 0.576$ and $r = 0.787$ as figured by Fisher's z-transformation. And on this index the mean is 1.883 ($\pm 0.190 = \pm 1.183/\sqrt{39}$).

If one is willing to rank the different readers by using this numerical measure of relative pathogenicity as an index, one finds for the twelve the following:

1.575 ± 0.196	1.600 ± 0.211	1.650 ± 0.204	1.675 ± 0.201
1.700 ± 0.173	1.875 ± 0.169	1.900 ± 0.179	1.925 ± 0.177
1.950 ± 0.179	2.125 ± 0.161	2.225 ± 0.198	2.400 ± 0.167

It was interesting to us to note that the order of these means for the different pathologists correlated well with the order of leniency appearing in their reading of all their own slides. This suggests that the distributions of their findings in their own hospitals are due in part to their habits of interpreting their slides and that interinstitution comparisons of their findings should not be made without some allowances for these differences, i.e., for what astronomers are wont to call the "personal equation" of the observer.

Classifying an individual slide is a "clinical" problem, as is any exercise of judgment about any individual case, whether pathologic, meteorologic, social, economic, medical, or psychological. Statistics deals with a population, and there may be such wide scatter about means or trends that the statistical findings may be of little use to the clinician. Moreover, no statistical study can include all the variables which may be acting. An important function of the clinician is to be alert to the special importance for the particular case of some variable which is not in the statisticians' findings for populations but may be controlling in the case before him; and, moreover, there are aspects of any clinical problem which may be sensed by the keen and experienced clinician but are not yet generally incorporated into standard statistical methods.[6]

Even had it been practicable, the co-operating pathologists should not have been forced to agreement with respect to standards of classification for this study at the danger of modifying their individual standards; for they were all experienced scientists, primarily responsible for long series of pathological determinations in their respective hospitals and should, therefore, be concerned with the continuity of standards in those series. Science is built upon the deductions one can draw from data freely assembled by different scientists with proper allowance for the inevitable differences between scientists. One can come to forced agreements, giving an artificial sense of security. The co-operating pathologists, after getting well into the study, were unanimously of the opinion that through it they were learning much that was new to them about pulmonary pathology and profiting greatly from their mutual contacts relative to it.

The original hope of Dr. Reimann's plan was to correlate the pathological findings with such environmental variables as amount and type of smoking, rural or urban residence, geographic location, occupation, etc. We hope that developments not ready for this meeting may later make it possible for us or others to extend this statistical discussion to some such matters; but in the short time available for the presentation of this paper we have been unable to exhaust even those materials and calculations which we consider germane to the topics here discussed.

[1] S. P. Reimann, "Pathologic-anatomic Study of Human Lungs: Tobacco Industry Research Committee Communication, June, 1957" (to be revised for publication). The agreement was to use approximately 15 sections in certain definite parts of the tracheobronchial tree. At least half of these sections were to have as much as 50 per cent of the mucosae intact. The average num-

ber of such sections proved to be about 12. This was not so detailed a study as that of Oscar Auerbach (*New Engl. J. Med.*, **256**, 97–104, January 17, 1957), who took a great many sections; but his study was at a single place and has reported only slightly over 100 cases, whereas this study involves over 1,000.

[2] Studies of "errors of observation" have long been made in the exact sciences on the assumption that there was something under observation which was far less variable than were the observer's possibilities of measuring it. In the medical and other inexact sciences the word "error" has sometimes been used where differences of information or of judgment in complicated situations are involved to such a degree that to attribute error to those who differ is undesirable. A few of the earlier studies of reliability are: R. C. Cabot, "Diagnostic Pitfalls Identified during a Study of Three Thousand Autopsies", *J. Am. Med. Assoc.*, **59**, 2295–2298, 1912; E. E. Southard and A. W. Stearns, "The Margin of Error in Psychiatric Hospital Diagnoses," *Boston Med. Surg. J.*, **171**, 895–900, 1915; Edwin B. Wilson and Julia Deming, "Statistical Comparison of Psychiatric Diagnoses in Some Massachusetts State Hospitals during 1925 and 1926," *Quart. Bull. Mass. Dept. Mental Diseases*, Vol. **11** (reprinted in *Schizophrenia: Statistical Studies from the Boston Psychopathic Hospital*, 1925–34, collected for private distribution by Dr. C. Macfie Campbell); C. R. Doering and A. F. Raymond, "Reliability of Observations in Psychiatric and Related Characteristics," *Am. J. Orthopsychiatry*, **4**, 249–257, 1934 (reprinted in *Schizophrenia*). E. E. Siegler, "Microdiagnosis of Carcinoma-in-Situ of the Uterine Cervix: A Comparative Study of Pathologists' Diagnoses," *Cancer*, **9**, 463–469, 1956; and C. C. Birkelo, W. E. Chamerlain, P. S. Phelps, P. E. Schools, D. Zacks, and J. Yerushalmy, "Tuberculosis Case Finding: Comparison of Effectiveness of Various Roentgenographic and Photofluorgraphic Methods," *J. Am. Med. Assoc.*, **133**, 359–366, 1947.

[3] If one does not wish to have so small a theoretical value as 7.4 in the chi-square test, one may make two divisions, viz., 0 and 1 and 2, 3, 4, and 5 with the result that chi-square is about 30 for 11 degrees of freedom, whereas the 1 per cent point is at 25.

[4] This shows that actually in practice the spread in the judgments stated is not just a borderline affair.

Another way to show the differences is by comparing the least severe and the most severe readings of the individual slides:

Least Severe	Most Severe 0	1	2	3	4	5	Totals
0	..	1	8	10	..	..	19
1	..	..	4	6	1	1	12
2	..	..	2	2	2	..	6
3	..	..	..	..	..	2	2
4	..	..	..	..	..	1	1
5	..	..	..	..	..	..	0
Total	0	1	14	18	3	4	40

Note the peculiar distribution of the frequencies of the differences in class between the least and most severe readings, namely, 2, 8, 18, 11, and 1, respectively, for differences of 0, 1, 2, 3, and 4 classes.

Still another way is to tabulate the 40 slides according to the number of different classes used in classifying each. The frequencies are 2, 11, 17, and 10, respectively, for the use of 1, 2, 3, and 4 classes. There is no slide for which all readers agreed that it was normal or that it was carcinoma.

[5] It would be a separate and probably involved study to determine how an index of pathogenicity should be made up. Naturally, "normal" would be taken as 0, and hyperplasia might be assigned the figure 1, but, apart from some intricate considerations of pathology and statistics, one could only assign classes 2, 3, 4, and 5 general values m, a, s, and c to stand for whatever might be the pathogenicities of those classes relative to hyperplasia. That $1 < m < a < s < c$ would be assumed because of the basic assumption that the classes are in increasing degree of pathogenicity. Consider a simple correlation table between two readers of the 40 slides such as:

Reader A	Reader B 0	1	2	3	4	5
0	4	1	2	..	..	..
1	6	2	4	..	..	..
2	1	1	13	..	..	..
3	..	..	2	1	..	..
4	..	..	..	..	..	..
5	..	..	..	..	..	3

It is clear that these readers agree in 23 of 40 cases and disagree in 17. The excess of Reader B over Reader A on the index would be

$$E = 1 + 2m + 4(m - 1) - 6 - m - (m - 1) - 2(a - m) = -8 + 6m - 2a,$$

and any values assigned to $m > 1$ and $a > m$ which make E positive will make Reader B severer than A. If we set $m - 1 = m' > 0$ and $a - m = a' > 0$, we have $E = -4 + 4m' - 2a'$ as the excess. If we plot a' as ordinate and m' as abscissa, we can say that, for any point (m', a') in the first quadrant which lies above the line $-4 + 4m' - 2a' = 0$, Reader B will be less severe than A, whereas, for any point below it, B will be more severe. If we take $m = 2$, $a = 3$, $m' = 1$, $a' = 1$ as we did, then $E = -2$ and B is the less severe.

[6] These matters have been discussed by P. E. Meehl in his book *Clinical versus Statistical Prediction* (Minneapolis: University of Minnesota Press, 1954) and before the 1956 convention of the American Psychological Association under the title "When Shall We Use Our Heads Instead of a Formula?" and in his article "Configurational Scoring," *J. Consulting Psychology*, **14**, 165–171, 1950.

THE INDEX TO THIS VOLUME HAS BEEN REMOVED FROM THIS POSITION AND PLACED AT THE BEGINNING OF THE FILM FOR THE CONVENIENCE OF READERS

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